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CONTENTS OF VOLUME 66

NO. 1, JANUARY, 1960

BROWN, J. H. U., FRANK S. LABELLA AND FRODE ULVEDAL
Studies on the Intracellular Localization of Anterior Pituitary Hormones by Means of Differentiai Centrifugation 1

ALIBRANDI, ANTONINO, GIANCARLO BRUNI, ALBERTO ERCOLI, RINALDO GARDI AND ALBERTO MELI
Factors Influencing the Biological Activity of Orally Administered Steroid Compounds: Effect of the Medium and of Esterification 13

WORTHINGTON, W. CURTIS, JR.
Vascular Responses in the Pituitary Stalk 19

KUMAOKA, SOICHI, WILLIAM L. MONEY AND RULON W. RAWSON
The Effect of Thyroxine Analogues on a Transplantable Mouse Pituitary Tumor .. 32

HOLLANDER, VINCENT P., MARY-LOUISE STEPHENS AND THELMA E. ADAMSON
The Effects of Estrogen on Uterine Phenol-Stimulated Oxidase 39

PERIČ-GOLIA, LUDVIK, KRISTEN EIK-NES AND RUSSELL S. JONES
Cortisol-4-C^{14} in the Adrenal of the Normal and Vitamin C Deficient Guinea Pig .. 48

MEAKIN, J. WILLIAM, WILLIAM H. TINGEY, JR., AND DON H. NELSON
The Catabolism of Adrenocorticotropic Hormone: The Stability of Adrenocorticotropic Hormone in Blood, Plasma, Serum, and Saline 59

MEAKIN, J. WILLIAM AND DON H. NELSON
The Catabolism of Adrenocorticotropic Hormone: Some Characteristics of an ACTH-Inactivating System in Plasma 73

ADLERCREUTZ, H. JR., E. DICZFALUSY AND L. ENGSTRÖM
Isolation and Identification of Bile Estrogens in Pregnant Women 80

GREENWALD, GILBERT S.
The Effects of Unilateral Ovariectomy on Follicular Maturation in the Hamster .. 89

GROSVENOR, CLARK E. AND CHARLES W. TURNER
Pituitary Lactogenic Hormone Concentration During Pregnancy in the Rat 96

LINDQUIST, BERTIL, ANN M. BUDY, FRANKLIN C. MCLEAN AND JOHN L. HOWARD
Skeletal Metabolism in Estrogen-Treated Rats Studied by Means of Ca^{45} 100

SAWYER, WILBUR H.
Increased Water Permeability of the Bullfrog (*Rana catesbiana*) Bladder *in vitro* in Response to Synthetic Oxytocin and Arginine Vasotocin and to Neurophysial Extracts from Nonmammalian Vertebrates 112

MEYER, J. E. AND J. T. BRADBURY
Influence of Stilbestrol on the Immature Rat Ovary and Its Response to Gonadotrophin 121

HORN, EUGENE H.
Reversibility of Thiouracil-Induced Inhibition of Interpubic Separation in Virgin Mice Given Thyroxine or Large Doses of Relaxin 129

NOTES AND COMMENTS

BOCCABELLA, ANTHONY V. AND RICHARD G. STUELKE
Changes in the Thyroid: Serum Radioiodide Concentration Ratio During the Estrous Cycle of the Rat 135

CHRISTENSEN, L. KORSGAARD
Triiodothyronine Uptake by Erythrocytes 138

REVESZ, CLARA, CLIFFORD I. CHAPPEL AND R. GAUDRY
Masculinization of Female Fetuses in the Rat by Progestational Compounds .. 140

LIPSCOMB, HARRY S. AND DON H. NELSON
Dynamic Changes in Ascorbic Acid and Corticosteroids in Adrenal Vein Blood after ACTH 144

ENDOCRINOLOGY STUDY SECTION
Announcements 147

NO. 2, FEBRUARY, 1960

BRODISH, A. AND C. N. H. LONG
Characteristics of the Adrenal Ascorbic Acid Response to Adrenocorticotrophic Hormone (ACTH) in the Rat 149

RUEGAMER, W. R., M. E. ALPERT AND F. R. SILVERMAN
Thyroxine Analogues and Cholesterol Metabolism: The Relative Effects of Various Thyroxine Derivatives on Growth, Oxygen Consumption and Tissue Cholesterol Concentrations in the Rat 160

EPSTEIN, ROBERT, LLOYD B. ERIKSON AND S. R. M. REYNOLDS
Reserpine and the Chronic Stress of Reduced Barometric Pressure in Female Rats.

Observations on Body Weight, Adrenal Weight and Histology and Hematocrit Values ... 167
BROWN, J. H. U. AND FRODE ULVEDAL
Studies on Ultracentrifuge Fractions of Anterior Pituitary with Special Reference to Adrenocorticotrophic Hormone ... 175
ROSENBERG, I. N., J. C. ATHANS AND A. BEHAR
Effect of Thyrotropin on the Release of Iodide from the Thyroid ... 185
EGDAHL, RICHARD H.
Adrenal Cortical and Medullary Responses to Trauma in Dogs with Isolated Pituitaries ... 200
MCCANN, S. M. AND PAUL HABERLAND
Further Studies on the Regulation of Pituitary ACTH in Rats with Hypothalamic Lesions ... 217
ROBERTSON, O. H. AND B. C. WEXLER
Histological Changes in the Organs and Tissues of Migrating and Spawning Pacific Salmon (Genus *Oncorhynchus*) ... 222
FREGLY, MELVIN J.
Production of Hypertension in Adrenalectomized Rats Given Hypertonic Salt Solution to Drink ... 240
BURGERS, A. C. J. AND CHOH HAO LI
Amphibian Ovulation *in vitro* Induced by Mammalian Pituitary Hormones and Progesterone ... 255
SHOEMAKER, WILLIAM C. AND THEODORE B. VAN ITALLIE
The Hepatic Response to Glucagon in the Unanesthetized Dog ... 260
RALPH, C. L. AND R. M. FRAPS
Induction of Ovulation in the Hen by Injection of Progesterone into the Brain ... 269
SANTOLUCITO, J. A., M. T. CLEGG AND H. H. COLE
Pituitary Gonadotrophins in the Ewe at Different Stages of the Estrous Cycle ... 273
ABRAHAM, S., P. CADY AND I. L. CHAIKOFF
Glucose and Acetate Metabolism and Lipogenesis in Mammary Glands of Hypophysectomized Rats in which Lactation was Hormonally Induced ... 280
INGLE, DAVID J.
Suppression of Steroid Diabetes by Walker Carcinoma in the Rat ... 289
NANDI, JEAN AND HOWARD A. BERN
Corticosteroid Production by Interrenal Tissue of Teleost Fishes ... 295
NOTES AND COMMENTS
PEREK, M. AND A. EILAT
Effect of Removal of Bursa Fabricii on Depletion of Adrenal Ascorbic Acid in ACTH-Treated Chicks ... 304
REICHLIN, SEYMOUR
Effect of Graded Doses of Anterior Pituitary Extract on Thyroid Weight and Thyroid/Serum Iodide Ratio in the Hypophysectomized Rat ... 306
SCHWARTZ, NEENA B. AND ARTHUR KLING
Stress-induced Adrenal Ascorbic Acid Depletion in the Cat ... 308
ZILE, MAIJA H.
Effect of Thyroxine and Related Compounds on Monoamine Oxidase Activity 311
DAVIS, RICHARD AND ROY V. TALMAGE
Evidence for Liver Inactivation of Parathyroid Hormone ... 312
CHANDRA, PRAKASH, LETICIA MENDIOLA AND R. DAVID COLE
The Effect of Prolactin on the Xanthine Oxidizing Activity of Pigeon Tissue 315
ANNOUNCEMENTS
The Endocrine Society, the 1960 Annual Meeting ... 317
1960 Laurentian Hormone Conference ... 321
International Society of Neurovegetative Research ... 323
Symposium on Uterine Implantation of Ova ... 325
Training Program for Steroid Biochemistry ... 326
Correction ... 326

NO. 3, MARCH, 1960

REICHLIN, SEYMOUR
Thyroid Response to Partial Thyroidectomy, Thyroxine and 2,4-Dinitrophenol in Rats with Hypothalamic Lesions ... 327
REICHLIN, SEYMOUR
Thyroid Function, Body Temperature Regulation and Growth in Rats with Hypothalamic Lesions ... 340
BONNYCASTLE, D. D. AND A. J. BRADLEY
Diphenylhydantoin and the Release of Adrenocorticotropic Hormone in the Albino Rat ... 355
FORTNER, JOSEPH G., PHYLIS A. GEORGE AND STEPHEN S. STERNBERG
Induced and Spontaneous Thyroid Cancer in the Syrian (Golden) Hamster ... 364

URIST, MARSHALL R. AND NANCY MARIE DEUTSCH
Osteoporosis in the Laying Hen 377
BROOKS, JOHN R., GEORGE J. HILL, II, ALBERT DE'SCOVILLE, JULIO PRIARIO, DIANE CROCKER AND HERBERT A. SELENKOW
A Study of the Survival and Function of Homografted Thyroid Tissue in Membrane Filter Chambers in the Rat 392
BAHN, ROBERT C., HENRY E. STORINO AND ROBERT W. SCHMIT
The Mass of the Zona Glomerulosa Following Complete Anterior and Posterior Hypophysectomy and Subtotal Removal of the Pars Tuberalis in the Dog 403
NEWCOMER, W. S. AND PAUL A. BARRETT
Effects of Various Analogues of Thyroxine on Oxygen Uptake of Cardiac Muscle from Chicks 409
LONGCHAMPT, JACQUES E., CARLOS GUAL, MAXIMILIAN EHRENSTEIN AND RALPH I. DORFMAN
19-Hydroxy-Δ^4-Androstene-3,17-Dione an Intermediate in Estrogen Biosynthesis 416
GOODMAN, A. DAVID
Studies on the Effect of Omega-methylpantothenic Acid on Corticosterone Secretion in the Rat 420
OMAE, T. AND G. M. C. MASSON
Compensatory Adrenal Hypertrophy as a Possible Cause of Metacorticoid Hypertension 428
EVANS, EDWARD S., LAWSON L. ROSENBERG AND MIRIAM E. SIMPSON
Relative Sensitivity of Different Biological Responses to Thyroxine 433
CORTELYOU, JOHN R., AILEEN HIBNER-OWERKO AND JOSEPH MULROY
Blood and Urine Calcium Changes in Totally Parathyroidectomized *Rana Pipiens* 441
MUNSICK, ROBERT A.
Effect of Magnesium Ion on the Response of the Rat Uterus to Neurohypophysial Hormones and Analogues 451
PÉRON, FERNAND G.
The Isolation and Identification of Some Adrenocorticosteroids Released by Rat Adrenal Tissue Incubated *In Vitro* 458
TULLNER, WILLIAM W.
Endocrine Effects of Methylenedianiline in the Rat, Rabbit and Dog 470
LAWRENCE, A. M. AND A. N. CONTOPOULOS
Ovarian Response to Administration of Hypophyseal Follicle-Stimulating or Human Chorionic Gonadotrophic Hormone in Alloxan Diabetic Rats 475
DAVIDSON, JULIAN M. AND WILLIAM F. GANONG
The Effect of Hypothalamic Lesions on the Testes and Prostate of Male Dogs ... 480
NOTES AND COMMENTS
FISKE, VIRGINIA M., G. KAREN BRYANT AND JANET PUTNAM
Effect of Light on the Weight of the Pineal in the Rat 489
RYAN, KENNETH J.,
Metabolism of 16-Ketoestrone by Human Placenta 491
TULLNER, WILLIAM W. AND ROY HERTZ
Effect of Intravenous o,p′ DDD on Plasma 17-Hydroxycorticoid Secretion in the Dog 494
ANNOUNCEMENTS
Gordon Research Conference 496
International Symposium on Growth 497
Inter-Society Cytology Council 499
Microcirculatory Conference, Inc. 499
The National Vitamin Foundation, Inc. 500
Correction 500

NO. 4, APRIL, 1960

MARTINI, L., A. PECILE, S. SAITO AND F. TANI
The Effect of Midbrain Transection on ACTH Release 501
BORLE, ANDRÉ B., GEORGE NICHOLS, JR. AND MORRIS J. KARNOVSKY
Some Effects of Adrenalectomy and Prednisolone Administraton on Extracellular Fluid and Bone Composition in the Rat 508
CALESNICK, B., V. R. ALTARELLI AND M. A. SPIRTES
Decrease in Aerobic Glycolysis of Erythrocytes Following the Continuous Administration of L-Triiodothyronine 517
GREIF, ROGER L.
Comparison of *In Vivo* and *In Vitro* Effects of Triiodothyronine 521
CLARK, IRWIN, WILLIAM BOWERS AND RAYMOND GEOFFROY
A New Method for the Bioassay of the Calcium Mobilizing Fraction of Parathyroid Extracts 527
MOSCONA, MALKA H. AND DAVID A. KARNOFSKY
Cortisone Induced Modifications in the Development of the Chick Embryo 533

DOUGHERTY, THOMAS F., MARTHA L. BERLINER AND DAVID L. BERLINER
11β-hydroxy Dehydrogenase System Activity in Thymi of Mice Following Prolonged Cortisol Treatment 550
DOCTOR, V. M.
Effect of Storage of NaI^{131} Solutions upon the Thyroid Uptake by Day-Old Chicks 559
YAMADA, TAKASHI AND MONTE A. GREER
The Effect of Bilateral Ablation of the Amygdala on Endocrine Function in the Rat 565
WOODS, M. C. AND M. E. SIMPSON
Purification of Sheep Pituitary Follicle-Stimulating Hormone (FSH) by Ion Exchange Chromatography on Diethyl-aminoethyl (DEAE)-Cellulose 575
STUCKI, J. C., A. D. FORBES, J. I. NORTHAM AND J. J. CLARK
An Assay for Anabolic Steroids Employing Metabolic Balance in the Monkey: The Anabolic Activity of Fluoxymesterone and its 11-Keto Analogue 585
WRIGHT, RUTH M. AND AARON B. LERNER
On the Movement of Pigment Granules in Frog Melanocytes 599
BEUTNER, ERNEST H. AND PAUL L. MUNSON
Time Course of Urinary Excretion of Inorganic Phosphate by Rats after Parathyroidectomy and after Injection of Parathyroid Extract 610
SAVARD, KENNETH AND JOSEPH W. GOLDZIEHER
Biosynthesis of Steroids in Stallion Testis Tissue 617
NOTES AND COMMENTS
GARREN, LEONARD AND ROY O. GREEP
Effect of Adrenal Cortical Hormones on Eruption Rate of Incisor Teeth in the Rat 625
PITT-RIVERS, ROSALIND
Synthesis and Physiological Activity of Some New Analogues of Thyroxine .. 628
BORIS, ALFRED AND EDGAR S. GORDON
The Effects of Adrenal Corticoids upon the Urinary Excretion of Ammonia in the Adrenalectomized, Acidotic Rat 630
CORRECTION 634
ERRATUM 634
ANNOUNCEMENTS
The Endocrine Society, the 1960 Annual Meeting 635
Program 1960 Meeting, The Endocrine Society 638
6th International Congress of Internal Medicine 644
Southwest Research Institute 645
Excerpta Medica Foundation 645
NEW BOOK REGISTER 645

NO. 5, MAY, 1960

GRAHAM, R. C. B., C. T. BEER AND R. L. NOBLE
The Inactivation of Pregnant Mares' Serum Gonadotrophin by Hydroquinones and Quinones 647
ADAMS, D. D.
A Comparison of the Rates at which Thyrotrophin and the Human Abnormal Thyroid Stimulator Disappear from the Circulating Blood of the Rat 658
REAVEN, G., A. F. SCHNEIDER AND E. P. REAVEN
Changes in the Metabolism of S^{35}-Labeled Sulfate Associated with Renal Calcification 665
BECKERS, C., M. J. SPIRO AND J. B. STANBURY
Preparation and Metabolism of Iodinated Peptides 669
HATFIELD, W. B., F. F. DAVIDOFF, J. E. ROSS AND D. F. TAPLEY
The Physiological Disposition in the Rat of the Acetic and Propionic Acid Analogues of Thyroxine and Triiodothyronine 676
HARAN-GHERA, NECHAMA, PETER PULLAR AND JACOB FURTH
Induction of Thyrotropin-Dependent Thyroid Tumors by Thyrotropes 694
LAZARUS, SYDNEY S. AND BRUNO W. VOLK
Effect of Diabetogenic Hormones on Blood Sugar and Pancreas of Sulfonylurea Treated Animals 702
WEINSTEIN, HOWARD, ROBERT M. BERNE AND HOWARD SACHS
Vasopressin in Blood: Effect of Hemorrhage 712
SAYERS, GEORGE AND NEIL SOLOMON
Work Performance of a Rat Heart-Lung Preparation: Standardization and Influence of Corticosteroids 719
CORRÊA, P. RIET, MARIA MARQUES AND E. M. WAGNER
Hyperglycemia Caused by the Oral Administration of Glucose in Turtles 731
DAVIDSON, JULIAN M., ALEXANDER N. CONTOPOULOS AND WILLIAM F. GANONG
Decreased Gonadotrophic Hormone Content of the Anterior Pituitary Gland in Dogs with Hypothalamic Lesions 735

GERSCHENFELD, H. M., J. H. TRAMEZZANI AND E. DE ROBERTIS
Ultrastructure and Function in Neurohypophysis of the Toad 741
DUNSEATH, WILLIAM J. R., FREDERICK P. FERGUSON AND DIETRICH C. SMITH
Effects of Moderate Decompression Stress upon Plasma Potassium and Glucose in the Adrenalectomized Cat 763
MULAY, A. S.
Corticosteroid Activity of a Transplantable Adrenocortical Carcinoma in Osborne-Mendel Rats 769
LOUGHLIN, R. E., MARY T. MCQUILLAN AND V. M. TRIKOJUS
The Influence of Thyrotrophic Hormone and Methyl-Thiouracil on the "Cysteinyltyrosinase" (and Proteinase) Activity of the Thyroid Gland of the Rat 773
NOTES AND COMMENTS
CROSSON, JOHN, JOHN FALCH AND SEYMOUR REICHLIN
Failure to Demonstrate TSH-Releasing Activity of Pitressin and Oxycocin .. 777
HAMOLSKY, MILTON W., DIANA B. FISCHER AND A. STONE FREEDBERG
Further Studies on the Plasma Protein-Thyroid Hormone Complex 780
BERNSTEIN, DONALD E.
Vascular Exclusion of the Liver: Its Influence on Glucose Metabolism 783
KOCHAKIAN, CHARLES D.
Protein Anabolic Action of Testosterone Propionate in Hyperthyroid-Castrated Rats 786
DANOWSKI, T. S. AND LORENNA PRICE
Organ Weights of Animals Maintained on Alleged Aphrodisiacs: "Pega Palo" and "Bois Cochon" 788
THE ENDOCRINE SOCIETY 791

NO. 6, JUNE, 1960

KOBAYASHI, HIDESHI AND AUBREY GORBMAN
Radioiodine Utilization in the Chick 795
URIST, MARSHALL R. AND NANCY MARIE DEUTSCH
Effects of Cortisone upon Blood, Adrenal Cortex, Gonads, and the Development of Osteoporosis in Birds 805
GUILLEMIN, ROGER
Steroidogenic Activities of Purified α, β, γ, δ-Corticotropins and Pepsin Degradation Products of β Corticotropin 819
ERIKSON, LLOYD B., S. R. M. REYNOLDS AND VINCENT J. DE FEO
Menstrual Irregularities in Rhesus Monkeys Elicited by Reserpine Administered on Selected Days of the Cycle 824
ROSENKRANTZ, HARRIS AND ROLAND O. LAFERTE
Further Observations on the Relationship Between Serotonin and the Adrenal ... 832
HERTZ, ROY
Gonadotropin and Adrenocorticotropin from Rat Pituitary Homografts as Manifested by Host Response to Chorionic Gonadotropin and Amphenone 842
KONO, TSUYOSHI, L. VAN MIDDLESWORTH AND E. B. ASTWOOD
Chemical Identification of Iodine-Containing Compounds in Human Serum 845
FREINKEL, NORBERT
Further Observations Concerning the Action of Pituitary Thyrotropin on the Intermediate Metabolism of Sheep Thyroid Tissue *in Vitro* 851
MUNSICK, ROBERT A., WILBUR H. SAWYER AND H. B. VAN DYKE
Avian Neurohypophysial Hormones: Pharmacological Properties and Tentative Identification 860
STASILLI, NEIL R., ROBERT L. KROC AND RAY EDLIN
Selective Inhibition of the Calorigenic Activities of Certain Thyroxine Analogues with Chronic Thiouracil Treatment in Rats 872
NOTES AND COMMENTS
BÉLANGER, LEONARD F.
Increased Mitotic Activity in the Glandular Stomach of the Rat Following Hypophysectomy 886
ANBAR, M., S. GUTTMANN AND Z. LEWITUS
The Accumulation of Fluoroborate Ions in Thyroid Glands of Rats 888
TESSARI, LUIGI
The Effect of Parathyroid Extract Upon Transaminase Activity in Metaphyseal Bone 890
CLIFTON, KELLY H. AND JACOB FURTH
Ducto-Alveolar Growth in Mammary Glands of Adreno-Gonadectomized Male Rats Bearing Mammotropic Pituitary Tumors 893

DZIUK, PHILIP J.
Inhibition and Synchronization of Mating in the Mouse by Oral Administration of Progestins 898
RINGLER, I. AND R. BROWNFIELD
The Thymolytic Activities of 16α, 17α Ketals of Triamcinolone 900

ANNOUNCEMENTS

The Endocrine Society 903
First International Congress of Endocrinology 905
The Fourth International Goitre Conference 906
Journal of Reproduction and Fertility, a new publication 906
First International Congress of Histochemistry and Cytochemistry 907
The American Institute of Oral Biology 907

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STUDIES ON THE INTRACELLULAR LOCALIZATION OF ANTERIOR PITUITARY HORMONES BY MEANS OF DIFFERENTIAL CENTRIFUGATION[1]

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ABSTRACT

Following the development of means for the separation of subcellular particles in anterior pituitary and the establishment of criteria of purity, each of the fractions was analyzed for hormone content and compared with results obtained by others. The fractions were assayed by a three-dose response method for TSH, ACTH, LH, and FSH. TSH was found in mitochondria, LH and STH in acidophilic granules, and FSH was distributed over several fractions. ACTH was found in the microsomes and in supernatant depending on the method of assay adopted. The predominant activity appeared in the indicated fraction, but there was scattered activity over several fractions. The results agree in the main with those of other investigators.

DESPITE the rapid progress made in the cytological identification of cells of the anterior pituitary, the site of production of many of the pituitary hormones has not been determined. Several groups (10, 11, 12) have attempted to isolate subcellular particles from pituitary cells and to determine the hormones associated with each fraction. Some confusion has arisen due to differences in method of separation of particles, to failure to designate fractions uniformly and to establish adequate criteria of homogeneity. A previous paper (1) from this laboratory demonstrated that it

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[1] This research was supported by Grant #A-897, National Cancer Institute, National Institutes of Health.

[2] This work was done during the tenure of a Research Fellowship of the American Heart Association.

[3] A portion of this work will be presented to fulfill the requirements of the Degree of Docotor of Philosophy at Emory University, Division of Basic Health Sciences.

was possible to obtain satisfactory separations of subcellular particles from the pituitary. The work to be reported here is concerned with further studies along this line.

MATERIALS AND METHODS[4]

A. *Obtaining glands.* Pig pituitaries were removed from the animals within 20 minutes after sacrifice. They were placed in glass vessels containing ice but were not allowed to freeze or to come in contact with the ice. After the connective tissue capsule was removed, the anterior portion was freed of the other lobes.

B. *Preparation of cell fractions.* Procedures were carried out in a cold box at 2 to 4° C. All equipment and solutions were precooled to that temperature. The tissue was homogenized within an hour and a half after the first gland was obtained, using a tissue grinder consisting of a glass grinding chamber and a fitted, motor-driven Teflon pestle. A 10% (w/v) homogenate was prepared in 0.25M sucrose containing 0.02% heparin (Upjohn). Ten to 20 grams of anterior lobe were processed at one time.

Nuclei and debris. The homogenate was placed in 50 ml. plastic tubes and centrifuged at 1200 rpm for 15 minutes. The pellet was rehomogenized in sucrose medium and recentrifuged twice. The final pellet, which was red or reddish-blue in color, was rehomogenized in sucrose and stored in the cold (Fraction I).

Acidophilic or secretory granules. The supernatant solution from Fraction I was centrifuged at 7000×g. (R. max) for 20 minutes in a Spinco Model L ultracentrifuge. The precipitate consisted of a firm white pellet with an overlying tan-colored, "fluffy" layer. The pellet and fluff were separately rehomogenized and centrifuged at 8000×g. for 15 minutes. The pellets were pooled, as were the fluffs, and each was rehomogenized and recentrifuged at 10,000×g. for 15 minutes. The milk-white pellets were pooled, rehomogenized and stored in the cold (Fraction II).

Mitochondria. The resuspended fluffy material and the supernatant solution were separately centrifuged at 34,000×g. for 15 minutes, resulting in firm, tan-colored pellets with an overlying fluffy layer. The firm pellet (Fraction 3) and the fluffy material (Fraction 3A) were rehomogenized separately and each was again centrifuged at 34,000×g. for 15 minutes.

Microsomes. The supernatant solutions from fractions 3 and 3A were centrifuged at 78,000×g. for 3 hours. The pellet produced by this centrifugation was a translucent red gel (Fraction 4). Contaminating mitochondria, if present, tended to form a central zone which could easily be removed and added to the main mitochondrial fraction.

Supernatant solution. The final supernatant solution (Fraction 5) was a perfectly clear, reddish solution.

C. *Morphological examination of cell fractions.* A few drops of the suspended material of each particulate fraction were mixed in 5 ml. of buffered 1% osmic acid containing 0.25M sucrose and fixed for 1 hour at 2 to 4° C. The tube containing the fixed suspension was centrifuged in a Spinco #40 head at 110,000×g. for 15 minutes. Sections were taken from several parts of the thin pellet for electron microscopical observation.

D. *Chemical analyses.* Aliquots of the suspension of cell fractions were separated into acid-soluble components, deoxyribose nucleic acid (DNA), and pentose nucleic acid (RNA) according to the method of Schneider (2). RNA was determined by the orcinol

[4] A description of the physical and biochemical properties of the anterior pituitary is in press (*J. of Biophy. and Biochem. Cytol.* **5**: 17. 1959). A portion of this data is summarized here to demonstrate the reliability of the assay data.

reaction for pentose (3); ribose values were taken as 38% of the weight of RNA. DNA determinations were not run routinely, since a few analyses showed this material to be present exclusively in the nuclear and debris fraction. Nitrogen was determined by Kjeldahl digestion and nesslerization (4). All chemical determinations were made in triplicate.

E. *Enzyme assays.* The following enzyme assays were done on each fraction.

Succinic dehydrogenase was assayed by the manometric method of Schneider and Potter (5) except that AMP(0.012M) and ATP(0.012M) were added to the reaction medium.

Alkaline phosphatase. The incubation medium contained 1.0 ml. of 0.1M boric acid-KCl-NaOH buffer at pH 9.8, 0.5 ml. of 0.2M beta-glycerophosphate (99.9% min., Eastman), and tissue suspension and water to 2.0 ml. The reaction mixture was placed in a 12×125 mm. test tube and incubated at 37° C for 10 minutes with occasional shaking. The reaction was stopped by the addition of 2.0 ml. of 10% trichloroacetic acid (TCA). The tube was centrifuged and inorganic phosphorus was determined on 2.0 ml. of the supernatant solution by the method of Dryer *et al.* (6).

Proteinase 8. Proteinase 8 was determined by the method of Adams and Smith (7). For all enzyme tests an aliquot of each fraction was taken so that approximately equivalent amounts of nitrogen were contained in the aliquots. All determinations were made in triplicate along with appropriate blanks.

F. *Hormone assay.* Fractions were administered in doses containing equal amounts of nitrogen per day using the following assay methods:

a. *TSH.* Thyrotrophic hormone was assayed by the uptake of radioiodine (I^{131}) in the hypophysectomized adult rat 24 hours after an injection of 1 microcurie of radioiodine following three days of injection with the test material. In some cases the animals used for STH assays were given radioiodine on the 10th day and autopsied on the 11th day. In all cases, the thyroid was dissected free from other tissue, dissolved in 10% NaOH, and counted in a well type scintillation counter.

b. *ACTH.* ACTH was assayed by the method of Sayers and Woodbury (8); by the decrease in the number of circulating eosinophils four hours following the intravenous injection of the material into hypophysectomized rats, and by the *in vitro* stimulation of steroid production by rat adrenals treated with the fractions (Saffran's procedure (14)) except that the more specific blue tetrazolium reaction (17) was used to detect the adrenal steroids in place of the non-specific ultraviolet method recommended by Saffran.

c. *LH.* The lutenizing hormone as assayed in immature and hypophysectomized rats of both sexes. The material was injected once daily for three days with autopsy on the 4th day. The weights of the seminal vesicles, testes, and ventral prostate were determined in the male, and the weights of the ovary and uterus were measured in the female.

d. *FSH.* Follicle stimulating hormone was assayed in immature female rats by the method of Steelman and Pohley (9) and in immature male rats by the increase in testes weight brought about by injecting the material daily for 3 days with autopsy on the fourth day.

At autopsy of animals used for the assay of growth hormone the accessory sex organs were weighed in order to provide additional data in corroboration of the more direct assays for FSH and LH.

e. *STH.* Growth hormone was assayed by the gain in weight of adult hypophysectomized rats given the test material for ten days with autopsy on the 11th day.

Animals used in the assays were carefully selected. All immature rats were 21–22 days of age at the start of injections. The hypophysectomized rats averaged 100 gm. in weight; the hypophysectomies were performed in this laboratory by approach through

the ear (personal communication Dr. C. N. H. Long's laboratory). All hypophysectomized animals were examined at autopsy for pituitary remnants. At least 6 animals were used for each dose of each fraction tested.

RESULTS AND DISCUSSION

The biochemical analyses of the subcellular particles of the anterior pituitary are presented in Table 1. RNA is found mainly in the microsomes, succinic dehydrogenase mainly in the mitochondria,[5] and alkaline phosphatase mainly in the microsomes. A similar distribution has been reported for particulate fractions of liver (5). The proteolytic enzymes of the pituitary with a pH optimum of 8 (Proteinase 8) are found mainly in the supernatant fraction, although a significant amount is also present in the microsomal fraction. The distributions of RNA and of enzyme activities are indicative of a fairly good separation of the cell components. It is not possible to obtain an absolutely clear cut separation but the differing patterns for the various fractions suggest that each is mainly made up of one component. The nuclear fraction, of course, contains nuclei, whole cells, and cell debris, and mildly reflects the composition of all components.

TABLE 1. CHEMICAL AND ENZYMATIC ANALYSIS OF THE SUBCELLULAR PARTICLES OBTAINED BY ULTRACENTRIFUGATION. THE DATA ARE AVERAGES OF FIVE CENTRIFUGATIONS

Fraction	Nitrogen % of total	RNA		Succinic dehydrogenase % of total	Alkaline phosphotase % of total	Alkaline proteinase % of total
		μg. mg. N	% of total			
I-Nuclei	12.6±1.8*	159±16	13.1±1.0	2.2±2.2	12.6± 1.7	2.2± 1.7
II-Acidophilic granules	16.0±2.4	46±28	4.8±2.0	10.6±7.0	5.0± 1.2	3.2± 1.9
III-Mitochondria	13.3±3.4	107±26	8.7±3.9	31.8±8.3	9.3± 4.9	7.0± 5.3
III_A-Light Mitochondria†	11.3±2.3	234±30	18.8±2.1	44.7±6.7	14.6± 5.8	12.1± 6.8
IV-Microsomes	11.0±1.6	507±107	41.3±8.0	5.0±1.3	24.5± 4.0	25.5±14.5
V-Supernatant	29.8±2.1	84±22	13.6±9.7	0.0±0.0	34.0±10.0	43.3±15.5
Recovery	97.5%		105.3%	57.3%	96.6%	98.0%

* Standard deviation.
† Fraction III_A is omitted from the Hormone Assay due to the fact that it does not contain hormone.

The acidophilic or secretory granules are not found in liver and comprise a separate physical entity in pituitary cells. These particles are rich in protein but contain little RNA and only small amounts of the enzymes measured.

The separation of components suggested by the chemical data is corroborated by the electronmicrographs of the fractions (Fig. 1). Parts A and B of the microphotographs are from sections of the whole gland showing the characteristic appearance of mitochondria, acidophilic granules, and basophilic granules. Part C is a section from a pellet of the acidophilic or secretory granule fraction showing the low order of contamination with other particulates. Part D, from the mitochondrial fraction, also shows relatively few structures of other types and demonstrates the characteristic cristae

[5] Succinic dehydrogenase is a major component of Fraction IIIA (*J. Biophys. & Biochem. Cytol.* **4**: 895. 1958). This fraction has not been included in this paper.

and double membranes of mitochondria. Part E, for contrast, is from the nuclear fraction, showing mitochondria, acidophilic granules, and "debris." Part F, from the microsomal pellets, shows a few structures that may be basophils and a mass of "cell reticulum." Supernatant fractions, not shown, are structureless.

It is interesting to note that the basophilic granules which are present in large numbers in the sections taken from whole cells are not found in any great number in any of the fractions. This is in confirmation of the observations of Herlant (12) who found no true basophils in any of his fractions. The basophils seem to dissolve in the medium and their contents may thus appear in the microsomal fraction and the supernatant. This may account

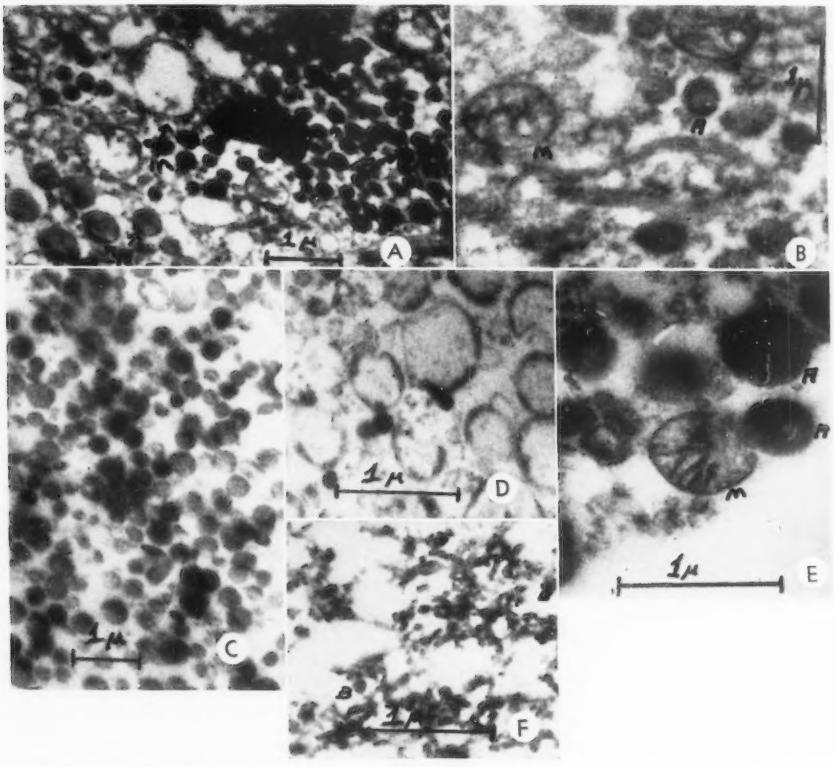

FIG. 1. Electronmicrophotographs of pig pituitary glands. A. Section of whole gland containing mitochondria (M), basophilic granules (B), and acidophilic or secretory granules (A). B. Another section of a gland showing further structural relationships. C. Pellet of acidophilic or secretory granules as separated in the ultracentrifuge. D. Section of pellet from Fraction 3, mitochondria. E. Section from nuclear fraction showing both mitochondria (M), and secretory granules (A). F. Microsomal fraction (Fraction 4). Note the absence of basophilic granules. Magnifications are indicated on each picture.

TABLE 2. THE ASSAY OF TSH AND STH IN THE ADULT HYPOPHYSECTOMIZED ANIMAL

All animals were injected once a day for ten days with the test fraction at the indicated doses. TSH was estimated by the uptake of radioiodine in the thyroid and STH by the growth rate of the animals. The I^{131} was administered i.p. 24 hrs. before autopsy. The data are averages of six centrifugations.

Fraction	Number animals	Dose, μg. N/day	Assay of STH, gm. gained/day	*M*†	Assay of TSH, counts/min. in thyroid × 1000	*M*
Control	48	—	−1.8±2.0*		2.8±0.5	
I-Nuclear	36	20	0.9±1.6‡		2.8±0.3	
	42	40	1.2±0.8	1.0	3.8±0.4	1.0
	36	60	2.2±1.1		5.6±0.7	
II-Acidophilic Granules	42	20	1.8±0.6		4.1±0.6	
	40	40	2.5±1.1	3.9	5.6±0.5	2.5
	49	60	3.2±0.8		9.1±0.5	
III-Mitochondria	48	20	1.4±0.2		7.9±0.4	
	49	40	2.1±0.6	2.9	10.8±0.4	13.1
	36	60	3.5±0.7		14.3±0.6	
IV-Microsomes	40	20	0.7±0.6		3.8±0.7	
	45	40	1.6±0.7	1.1	4.3±0.5	3.4
	49	60	1.5±0.5		3.8±0.4	
V-Supernatant	50	20	1.0±1.0		5.4±0.3	
	49	40	1.7±0.8	1.6	6.1±0.3	8.1
	48	60	3.8±1.2		8.0±0.4	
STH-Standard	118	3	2.3±0.7			
		15	2.8±0.9			
TSH-Standard	18	16			6.2±0.4	

* Standard deviation.

† $M = KI\ D/B$

Where $K = \sqrt{\frac{\text{Cases}}{\text{no. doses}}}$; $2D = (U_1 + U_2) - (S_1 + S_2)$

$I = \log \frac{\text{High dose}}{\text{low dose}}$; $2B = (S_2 + U_2) - (S_1 + U_1)$

U = Unknown dose-response

S = Known dose-response

‡ Although it is not strictly true, parallelism between dose-response curves is assumed for purposes of approximate calculation.

for the high proportion of total nitrogen found in the latter fraction (Table 1) as well as for some of the hormonal activity of this fraction to be discussed below.

The data of Table 1 and Figure 1 taken together indicate that a good separation of the major cellular components has been achieved, that each fraction is relatively "clean," and that any hormonal activity found mainly in a given fraction may reasonably be assumed to be localized in the particle characterizing that fraction. A perfect separation cannot be expected because of the variation in size or density of a given type of particle, be-

cause of the fragility or solubility of the particles, and because some activities may be leached out of a given particle or appear in the supernatant.

The data obtained upon assay of each of the fractions for hormone content are presented in Tables 2, 3, and 4. In Table 2 data are given for a three dose range of each fraction but only the two lower doses were used in calculating the relative potency of each fraction as compared to Fraction I (log dose ratio, M) because examination of the dose-response curves suggests that interaction (synergism) between hormones alters the dose-response relationship in the higher dose ranges. For example, the fraction containing STH in some quantity also contains TSH in larger amounts (Fraction III). The augmentation of the STH response by thyroid hormone production stimulated by TSH is demonstrated by the sudden increase in the slope of the dose-response curve at the top dose in this fraction. Similarly, the STH response is decreased in the presence of excessive amounts of ACTH (Fraction IV) and this results in a definite flattening of the STH dose-response curve in this fraction. The same effect can be seen in Table 5 where the log-dose-response for STH based on thymus weight has a positive slope in the presence of those fractions associated with STH and a negative slope in those fractions associated with ACTH with an intermediate slope between the two extremes. By the same analysis, it could be surmised that the growth response shown in Fraction III where augmenta-

TABLE 3. THE ASSAY OF ACTH BY THE EOSINOPHIL DEPLETION RESPONSE, THE ASCORBIC ACID DEPLETION METHOD, AND THE SAFFRAN METHOD IN VARIOUS FRACTIONS RESULTING FROM PITUITARY ULTRACENTRIFUGATION

At least 10 animals were used in each group. In the *in vitro* stimulation method, 1000 micrograms of nitrogen of the desired fraction were incubated with adrenals from four rats using Saffran's method of one hour of preincubation followed by 2 hours of incubation with the desired substance.

Fraction	Dose, μg. N	% decrease in adrenal ascorbic acid	M*	% decrease in eosinophils	M	*In vitro* steroid production, μg. 100 mg. adrenal hr.		
						E-240	BTZ	Ratio E240 BTZ
Homogenate	10	12±4		33± 8		7.5±1.1	3.9±1.7	1.92
	20	25±6		59± 5				
Fraction I	10	14±5		26± 7		6.8±0.7	3.9±0.1	1.72
	20	20±1	1.00	39± 6	1.0			
	30	28±6		44± 6				
Fraction II	10	6±3		25± 6		6.1±2.1	4.0±1.8	1.51
	20	10±3	0.10	43± 6	0.9			
	30	25±5		36± 4				
Fraction III	10	13±3		39± 9		8.7±1.7	5.5±1.7	1.58
	20	16±3	0.25	59± 9	3.5			
	30	19±5		65±14				
Fraction IV	10	11±1		37± 7		9.1±1.0	5.4±1.7	1.68
	20	25±4	1.00	41± 7	1.0			
	30	35±6		46± 6				
Fraction V	10	8±3		14± 6		36.2±6.0	18.7±6.0	1.89
	20	14±4	0.50	31± 2	1.0			
	30	25±5		34± 2				
ACTH std.	0.10	24±4		24± 4		2.8±0.2	1.5±0.06	1.82
	0.20	36±4		51±12		4.3±0.7	2.2±0.12	1.96
Hypox Control 16	—	0		1± 2		0	0	

* M, the log dose ratio is calculated by the method indicated in the footnote to Table 2.

tion with TSH must occur, may be increased out of proportion to the true amount of hormone present. TSH, on the other hand, is not greatly augmented by other hormones and the localization is much sharper.

With these limitations in mind, localization of the pituitary hormones can be discussed. TSH appears to be sharply confined to the mitochondrial fraction. The presence of TSH in the supernatant solution may reflect the solubility of the material, or the fragility of the mitochondria. If the pos-

TABLE 4. THE ASSAY OF PITUITARY GONADOTROPHINS IN IMMATURE AND HYPOPHYSECTOMIZED ANIMALS

At least six animals were used at each dose level in each fraction. The results summarize the data for approximately 50 animals at each dose level in 7 centrifugations. The fractions correspond to those previously reported.

Fraction	Dose, μg.N/day	Seminal vesicle, wt. (mg.)	*M*†	Testes weight, wt. (mg.)	*M*	Ventral prostate, wt. (mg.)	*M*	Ovary wt. (mg.)	*M*	Uterus, wt. (mg.)	*M*
Immature Animals											
I	20	18 ± 3	1.00	180 ± 24*	1.00	37 ± 6	1.0	21 ± 4	1.0	22 ± 2	1.0
	40	27 ± 4		195 ± 18		40 ± 2		22 ± 2		26 —	
II	20	43 ± 5	6.6	219 ± 21	3.25	40 ± 4	1.1	25 ± 1		25 ± 2	1.8
	40	56 ± 5		268 ± 14		39 ± 5		37 ± 3	2.9	31 ± 3	
III	20	18 ± 4	1.0	222 ± 25	4.5	47 ± 4	2.5	28 ± 2	2.3	28 ± 2	3.4
	40	25 ± 6		238 ± 19		48 ± 6		34 ± 4		32 ± 2	
IV	20	20 ± 4	1.18	205 ± 32	2.6	41 ± 8	2.6	18 ± 3	0.9	20.0 ± 5	1.3
	40	28 ± 4		239 ± 26		45 ± 3		23 ± 3		30.0 ± 3	
V	20	24 ± 3	1.71	195 ± 18	2.6	44 ± 6	2.6	20 ± 5	1.8	18 ± 2	0.32
	40	34 ± 4		220 —		52 ± 7		30 ± 4		20 ± 4	
Control		20 ± 4		195 ± 18		14 ± 8		22 ± 3		20 ± 2	
Hypophysectomized											
I	20	37 ± 4	1.0	550 ± 66		29 ± 6		41 ± 5		26 ± 2	1.0
	40	38 ± 4						77 ± 4		31 ± 1	
II	20	60 ± 8	18.0	683 ± 56		35 ± 7		32 ± 6		43 ± 3	7.4
	40	78 ± 9						35 ± 5		52 ± 4	
III	20	38 ± 7	2.1	548 ± 71		18 ± 6		38 ± 4		34 ± 4	2.6
	40	50 ± 9						44 ± 3		49 ± 3	
IV	20	38 ± 9	2.2	629 ± 64		24 ± 4		39 ± 4		30 ± 4	1.3
	40	57 ± 7						36 ± 4		33 ± 2	
V	20	26 ± 6	0.3	683 ± 58		20 ± 5		28 ± 3		28 ± 2	1.0
	40	32 ± 4								29 ± 3	
Control				368 ± 44		20 ± 4		28 ± 3		17 ± 2	

* Standard deviation.
† *M*, the log dose relation is calculated by the method indicated in the footnote to Table 2.

sible augmentation of STH by TSH is taken into account, growth hormone appears to be localized rather sharply in the secretory granules (Fraction II). The failure to find large amounts of growth hormone in the supernatant may be a reflection of the more stable structure of these granules.

The localization of ACTH is not as clear cut as that of STH or TSH (Table 3). As determined by the ascorbic acid depletion test ACTH is present in Fractions I and IV; the eosinopenic response test reveals widely scattered activity with greater response in Fraction III; and the Saffran *in vitro* steroid production test discloses activity in Fraction V.

Examination of the data with respect to the eosinophil response suggests that this test is relatively non-specific. All fractions assayed are essentially equal in ACTH on the basis of this test with the exception of Fraction III. Equal quantities of protein produce equal responses and this may suggest a response which is dependent upon a nonspecific protein rather than ACTH.

There is no ready explanation of the rather clear cut differences in re-

TABLE 5. OTHER ORGAN WEIGHTS

The organ weights of hypophysectomized and of immature rats treated with the indicated fractions. All other autopsy data are presented in the preceding tables. Results are averages of 6 centrifugations.

Fraction	Dose mg. N	No. animals	Immature			Hypox		
			Thymus	*M*†	Adrenal	Thymus	*M*†	Adrenal
I	20	36	61 ±21*	1.0	12 ±2.1	387 ±96	1.00	23.8 ±1.2
	40		123 ±18			447 ±101		27.3 ±1.6
II	20	32	84 ±24	1.32	11.5 ±2.1	434 ±84	3.21	21.2 ±1.9
	40	35	150 ±29		12.0 ±1.9	434 ±92		29.3 ±2.1
III	20	36	64 ±21	1.00	13.3 ±1.8	408 ±111	negative	21.4 ±1.6
	40	34	120 ±33		12.2 ±1.4	395 ±162	slope	21.3 ±1.6
IV	20	36	45 ±22	0.65	9.8 ±1.9	425 ±121	negative	22.7 ±1.9
	40	35	77 ±21		10.2 ±2.1	278 ±92	slope	22.7 ±1.4
V	20	36	75 ±24	0.64	9.8 ±2.2	412 ±102	negative	23.0 ±2.1
	40	36	74 ±26		10.9 ±2.1	373 ±93	slope	22.4 ±2.0
Control		24	75 ±25		10.1 ±2.1	253 ±93		19.1 ±1.9
STH	15	20	121 ±29		2.1 ±2.3			
ACTH	15	20	38 ±11		14.5 ±2.4			

* Standard deviation.
† *M*, the log dose relation is calculated by the method indicated in the footnote to table 2.

sponse in the two tests which are said to be relatively specific. The ascorbic acid depletion test shows a clear maximum in Fraction IV while the equally specific Saffran Test has a maximum in Fraction V. It is possible, but by no means proven, that two ACTH's exist as suggested by Stack-Dunne and Young (16). Further work is now in progress along these lines.

Although the eosinophil response may be non-specific, there is no evidence for non-specific *in vitro* stimulation of the adrenal as claimed by Roberts (14). The *in vitro* response is clear cut and since equal amounts of nitrogen were used in each flask, differences between fractions cannot depend upon this factor.

Assays for gonadotrophins are complicated by the marked synergism of LH and FSH (Table 3). Determination of LH by the response of the seminal vesicle weight in both hypox and immature animals reveals the pres-

ence of the hormone in Fraction II (secretory granules). Assay of FSH increase of testes weight is not conclusive. The use of Steelman's method of augmentation with HCG in order to override the LH activity and reveal FSH in the various fractions resulted in the appearance of FSH in all fractions. It would appear that the evidence favors localization of the gonadotrophins in Fraction II and the evidence is stronger for LH in this fraction than for FSH. The relatively non-specific uterine weight response test disclosed gonadotrophic activity in Fractions II and III.

Other organ weights (Table 5) reveal little of interest. There is an increase in thymus weight in those fractions containing STH and a decrease in weight in those fractions associated with ACTH as might be predicted from the action of the hormones on this organ. It is interesting that the decrease in thymus weight occurs to the largest extent in that fraction (#4) which contains ACTH as measured by ascorbic acid depletion. It is perhaps possible that the *in vitro* stimulation of the adrenal gland depends upon a "released" form of ACTH which is present in the supernatant while other less direct measures of adrenal action do not depend upon this factor. The adrenal weights do not add materially to the picture. There is no significant increase in weight of the gland in any fraction tested.

No attempt has been made to assay the fractions for lactogenic hormone. A method of assay is under study and data will be reported at some future time.

The work reported here has been concerned with pig pituitary but beef and sheep pituitaries have also been centrifuged by the same method. quantitative but not qualitative differences exist between species.

Some of the confusion in the literature can be resolved by this work. Reid and Segaloff (10), for example, reported that growth hormone was distributed over all fractions. It is possible to obtain this result by poor separation (and no criteria for particle homogeneity were given) which would result in widespread activity. Examination of Reid's data reveals such a wide spread, and it also shows activity to be present in the mitochondrial fraction as reported here. Ziegler and Melchior (11) found growth hormone in a "large granule" fraction which probably contained both mitochondria and acidophilic granules as judged from their speeds and times of centrifugation. They also found 50% of FSH and all of LH in this same fraction. Much of the LH appears in the acidophilic fraction in our centrifugations and since this is a part of Ziegler's "large granule" fraction, it is not surprising that we again agree with reported results. Herlant (12) has reported the separation of a pure white acidophilic fraction (?) which may correspond to the same fraction obtained in our separations and found ACTH, TSH, and LH in this fraction. However, he stated erroneously that mitochondria were negligible in amount. Herlant also failed to establish the homogeneity of his fractions and from the centrifuge data he must have had mitochondria contaminating the acidophilic granules. McShan and

Meyer (13) examined subcellular fractions of rat pituitary for gonadotrophic activity and found it to be in the "large" granules (mitochondria and acidophilic granules?) and in the "small" granule fraction (microsomes?). Herlant also found FSH in the basophilic or microsomal layer. We have found FSH to be present in most of the fractions tested. No true basophilic granules are present in the fractions although they are present in electron micrographs from whole cells. The basophilic granules apparently dissolve in the centrifuge medium, and this may explain some activity in the supernatant.

The major discrepancies between investigators appear to be a matter partly of terminology and partly of variations in methods of centrifugation. The separation of subcellular particulates into "large" and "small"

TABLE 6. THE LOCALIZATION OF HORMONAL ACTIVITY IN VARIOUS SUBCELLULAR FRACTIONS OF PITUITARY AS REPORTED BY VARIOUS AUTHORS

	STH	ACTH	FSH	LH	TSH
Acidophilic granules	6	2		[2], 6	5
Mitochondria	[1], [4], 5, 6		1	[1]	[2], 6
Microsomes	4	6	2, 3, 6		
Supernatant	1, 4	6	1, 5, 6	1, 5	

Bracketed fractions indicated assay on fractions containing "large" granules which probably had both acidophilic granules and mitochondria present.

1. Ziegler and Melchior (11)
2. Herlant (12)
3. McShan and Meyer (13)
4. Reid and Segaloff (10)
5. Brown and Hess (1)
6. This study

granules results in a large granule fraction containing both acidophilic granules and mitochondria. Assays on this fraction will give positive results for hormones present in both types of particles and may result in augmentation. Chemical identification is not always adequate. While it is true that most of the succinoxidase, for example, is in the mitochondria, a mixture of acidophilic granules and mitochondria would give essentially the same distribution of the enzyme although total activity per milligram of nitrogen would be altered. Only by a combination of histological, biochemical, and enzymatic tests can the criteria of purity of a given fraction be established. This has been the aim of work from this laboratory.

Another point remains to be resolved. Ziegler and Melchior found considerable hormonal activity in the supernatant and Reid was partially able to confirm their result for growth hormone. On the other hand, McShan (13) demonstrated that varying methods of treatment of the tissue would result in different amounts of gonadotrophin appearing in the supernatant. We find less hormonal activity in the supernatant and this may be attributed to two possible reasons. First, the greatest of care was taken in homogenizing and handling the tissue which may have prevented rupture

of many particles and secondly, the use of heparin in the centrifugation media resulted in better preservation of particulates and possibly less release of hormone into the supernatant.

As a summary of the current information, the results of various workers are presented in tabular form (Table 6). Activity of hormones is indicated by qualitative rather than quantitative measure. It is immediately apparent that a wide area of agreement exists. Most workers have found growth hormone in mitochondria or "large" granule fraction. Agreement seems to be widespread that FSH appears in "small" granules or supernatant and that LH appears in "large" granules, probably in the acidophils. TSH appears in the mitochondria. The small area of disagreement with previous work in this laboratory (1) is due to imperfect separation and naming of fractions and this older work is subject to many of the objections outlined above.

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REFERENCES

1. Brown, J. H. U. and Melvin Hess: *Amer. J. Physiol.* **88**: 25. 1957.
2. Schneider, W. C.: *J. Biol. Chem.* **164**: 7474. 1946.
3. Brown, A. H.: *Arch. Biochem.* **11**: 269. 1946.
4. Johnson, M. J.: *J. Biol. Chem.* **137**: 576. 1941.
5. Schneider, W. C. and V. R. Potter: *J. Biol. Chem.* **149**: 217. 1949.
6. Dryer, R. L., A. R. Tammes, and J. I. Routh: *J. Biol. Chem.* **225**: 177. 1957.
7. Adams, E. and E. L. Smith: *J. Biol. Chem.* **191**: 651. 1951.
8. Sayers, S. A. and L. A. Woodbury: *Endocrinology* **42**: 379. 1948.
9. Steelman, S. L. and F. M. Pohley: *Endocrinology* **53**: 604. 1953.
10. Reid, E. and A. Segaloff: *Proc. Soc. Exp. Biol. and Med.* **97**: 187. 1958.
11. Ziegler, D. M. and J. M. Melchior: *J. Biol. Chem.* **222**: 721. 1956.
12. Herlant, M.: *Annal. Endocrinol.* **13**: 611. 1952.
13. McShan, W. H. and R. K. Meyer: *Endocrinology* **50**: 294. 1952.
14. Roberts, S.: Hormones in Blood, Little, Brown Co., Boston, 1957.
15. Saffran, M., Grab, B. and M. J. Bayliss: *Endocrinology* **50**: 639. 1952.
16. Stack-Dunne, M. P., and F. G. Young: *J. Endocrinol.* **7**: IXVI. 1951.
17. Brown, J. H. U. and A. Anason: *Endocrinology* **62**: 103. 1958.
18. Emmens, C. W.: Hormone Assay, Academic Press, Inc. 1950.

FACTORS INFLUENCING THE BIOLOGICAL ACTIVITY OF ORALLY ADMINISTERED STEROID COMPOUNDS: EFFECT OF THE MEDIUM AND OF ESTERIFICATION

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ABSTRACT

The influence of the medium (aqueous or oily) and of esterification on the biological activity of some steroid hormones given orally to the rat has been studied.

Androgenic compounds were assayed by their effects on seminal vesicle, ventral prostate and levator ani weights in castrated male rats and cortisone-like substances by their effect on thymus, spleen, adrenal and granuloma weights in intact female rats.

All tested compounds exhibited an increase in specific activity when administered orally in oily solution instead of aqueous suspension.

The steroid compounds (i.e. androstanolone and in some instances prednisone) esterified with short chain fatty acids also exhibited a greater specific activity than the free steroid compounds.

Some hypotheses have been advanced to elucidate possible mechanisms by which lipidic solvent or esterification may potentiate the specific activity of orally administered steroid compounds.

MANY studies have been done on the factors influencing the intestinal absorption of cholesterol, such as the presence of bile and pancreatic juice, the addition of bile salts, the presence and nature of fats, the administration of cholesterol in esterified form, the simultaneous administration of other sterols, etc. (see 1–4). Little is known, however, about the influence of these factors on the intestinal absorption of steroid hormones. Apart from some studies on estrogens (5, 6), only the role of bile has been extensively examined in this respect. After the early works of Selye (7) and Hoffman *et al.* (8), the researches of Doisy and his coworkers (9–14) demonstrated a total enteric absorption of several steroids even in the absence of bile. Previously Wells *et al.* (15) had found that the biological activity of orally administered testosterone was not increased by the addition of biliary salts.

As a first approach to the problem, experiments were carried out in order to determine if either the form of steroid used (esterified or free) or the steroid vehicle (lipidic or aqueous) could influence the biological activity of some orally administered steroid hormones in the rat. As far as the latter

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question is concerned, preliminary investigations in man (16) suggested a greater activity of some orally administered steroids when given in oily solution.

MATERIALS AND METHODS

The steroids tested were some testosterone esters, free androstanolone (dihydrotestosterone, androstan-17β-ol-3-one) and some of its esters, 17α-methyl-testosterone, free prednisone and prednisolone and some of their esters. They were administered once daily by stomach tube either in oily solution (made by dissolving the steroid with gentle heating in sesame oil) or in aqueous suspension (made by means of a grinder). As suspending vehicle, an aqueous solution of sodium chloride (0.9%), carboxymethylcellulose (0.5%), Tween 80 (0.4%) and benzyl alcohol (0.9%) was used. Microscopic examination of the suspension showed that the diameter of the suspended particles was not greater than 10μ. In each experiment the substances to be compared were administered in equimolar doses (see tables). For purpose of comparison some steroids were also administered subcutaneously.

A) *Androgenic and myogenic activity.* The assay was performed according to the method described by Hershberger *et al.* (17). Male albino rats from our stock, weighing about 50 gm. and maintained on a standard diet, were used. Animals were castrated under ether anesthesia. The treatment with steroids was started on the day of castration and lasted for seven consecutive days. On the morning of the 8th postoperative day, the animals were killed by a blow on the head and the weight of ventral prostate, levator ani and seminal vesicles (without coagulating gland and devoid of fluid) determined to the nearest 0.5 mg.

B) *Cortisone-like activity.* The assay was performed according to the method described by Cresseri and Meli (18) with some procedural modifications. Female albino rats from our stock, weighing about 50 gm. and maintained on a standard diet, were used. Under ether anesthesia, a little disk (5 mm. in diameter and 5 mm. in thickness) of 5% agar was inserted subcutaneously into the dorsal anterior medial region of each animal. The treatment with substances to be tested started on the morning of the postoperative day and lasted for six consecutive days. On the morning of the 8th postoperative day, the animals were killed by ether asphyxiation and the subcutaneous implant carefully excised. The newly formed connective tissue was isolated and freed from the agar residues, dried on filter paper and immediately weighed on a torsion balance. The weight of the thymus, spleen and adrenals was determined to the nearest 0.5 mg.

RESULTS

The data obtained by treating animals with testosterone esters and androstanolone (free and esterified) are reported in Table 1. The results of 17α-methyl-testosterone treatment are reported in Table 2. The data concerning prednisone, prednisolone and their esters are found in Table 3.

Our experiments show that nearly all the tested compounds belonging to the androgen and some of cortical series show a greater degree of specific activity when orally administered in oily solution than when administered in aqueous suspension by the same route.

A more favourable anabolic index is obtained by oral administration of androgens, especially in aqueous suspension, than when they are administered subcutaneously.

TABLE 1. ANDROGENIC AND MYOGENIC ACTIVITY OF SOME FREE AND ESTERIFIED STEROIDS GIVEN ORALLY EITHER IN AQUEOUS SUSPENSION OR IN OILY SOLUTION

Treatment	Daily dose (20 μMoles) (mg.)	Number of animals	Organ weight (mg./100 gm. body weight)†		
			Seminal vesicles	Ventral prostate	Levator ani
Controls	—	160	6.0±0.1	14.5±0.3	19.6±0.3
Testosterone formate (A)	6.320	5	11.7±1.0	41.6±4.5	29.4±1.7
Testosterone formate (B)	6.320	5	*36.4±1.5*	*105.2±8.5*	*47.4±2.2*
Testosterone acetate (A)	6.600	5	17.6±1.4	65.6±5.8	35.9±2.4
Testosterone acetate (B)	6.600	5	*39.5±2.9*	102.2±8.0	*52.2±1.8*
Testosterone propionate (A)	6.880	5	13.8±0.8	41.6±4.7	30.4±1.3
Testosterone propionate (B)	6.880	5	*51.6±4.6*	*99.2±8.5*	*47.8±1.8*
Testosterone valerate (A)	7.440	5	13.8±1.7	43.8±5.0	25.8±3.2
Testosterone valerate (B)	7.440	5	*26.4±0.7*	*78.2±7.9*	*38.2±1.0*
Androstanolone (A)	5.800	10	9.7±0.4	49.6±3.9	27.8±1.9
Androstanolone (B)	5.800	10	*13.4±1.0*	*74.7±6.0*	*34.6±1.9*
Androstanolone formate (A)	6.360	10	16.6±1.4*	59.7±3.8	34.8±2.0*
Androstanolone formate (B)	6.360	10	*41.0±5.0**	*95.8±8.0*	*51.5±2.2**
Androstanolone acetate (A)	6.640	10	19.2±1.6*	61.4±5.0	35.6±2.0*
Androstanolone acetate (B)	6.640	10	*45.4±3.6**	*98.0±4.6**	*54.0±1.8**
Androstanolone propionate (A)	6.920	10	19.4±1.4*	58.5±5.0	36.6±2.0*
Androstanolone propionate (B)	6.920	10	*28.0±1.8**	*89.9±6.5*	*43.8±2.5**
Androstanolone oenanthate (A)	8.040	8	12.0±0.9*	49.6±3.9	32.4±2.7
Androstanolone oenanthate (B)	8.040	8	*21.4±1.2**	*82.6±5.4*	*49.2±2.3**
Steroids given by subcutaneous route (for comparison purposes)					
	(1 μMole)				
Testosterone (A)	0.288	10	52.3±2.9	108.0±4.8	58.7±2.6
Androstanolone (A)	0.290	10	49.3±1.0	131.9±7.4	78.0±2.7
Androstanolone (B)	0.290	10	38.0±2.6	114.2±6.9	68.9±2.3
Testosterone propionate (A)	0.344	10	40.7±3.3	96.6±5.5	52.3±3.5
Testosterone propionate (B)	0.344	10	66.4±2.4	136.5±6.2	76.0±3.0

(A) Daily dose in 0.5 cc. aqueous suspending vehicle.
(B) Daily dose dissolved in 0.2 cc. sesame oil.
† Mean values and standard errors are presented. In the upper part of the table, when the values are significantly different ($P<0.05$) from appropriate controls, they are printed in italics (steroids in oily solution versus aqueous suspension) or marked by asterisk (esterified steroids versus free ones). Actually most of values are significant at $P<0.01$.

TABLE 2. ANDROGENIC AND MYOGENIC ACTIVITY OF 17α-METHYL-TESTOSTERONE GIVEN ORALLY EITHER IN AQUEOUS SUSPENSION OR IN OILY SOLUTION

Treatment	Daily dose (mg.)	Number of animals	Organ weight (mg./100 gm. body weight)†		
			Seminal vesicles	Ventral prostate	Levator ani
Controls	0	160	6.0±0.1	14.5±0.3	19.6±0.3
Methyltestosterone (A)	1	8	16.7±1.3	75.0±6.0	33.2±1.3
Methyltestosterone (B)	1	8	*26.7±2.9*	91.4±8.7	*46.0±2.0*
Methyltestosterone (A)	2	10	23.4±1.4	91.2±6.5	37.2±1.6
Methyltestosterone (B)	2	10	*45.7±2.0*	*126.7±5.0*	*53.9±1.6*

(A) Daily dose in 0.5 cc. aqueous suspending vehicle.
(B) Daily dose dissolved in 0.2 cc. sesame oil.
† Mean values and standard errors are presented. When the values are significantly different ($P<0.01$) from appropriate controls, they are printed in italics (steroids in oily solution versus aqueous suspension).

TABLE 3. THYMOLYTIC AND ANTI-INFLAMMATORY ACTIVITY OF SOME FREE AND ESTERIFIED STEROIDS GIVEN ORALLY EITHER IN AQUEOUS SUSPENSION OR IN OILY SOLUTION

Treatment	Daily dose (0.5 μMoles) (mg.)	Number of animals	Organ weight (mg.)†			
			Thymus	Spleen	Adrenals	Granuloma
Controls	—	50	177.8± 7.2	349.5±13.0	13.3±0.4	66.0±1.8
Prednisone (A)	0.179	10	94.4± 9.5	305.6±24.0	12.6±0.7	59.4±4.7
Prednisone (B)	0.179	10	74.0±11.6	253.7±19.7	11.3±0.6	49.9±2.6
Prednisone formate (A)	0.193	10	92.4±10.8	320.3±35.6	11.3±0.8	52.6±5.3
Prednisone formate (B)	0.193	10	*60.6± 9.4*	*224.5± 8.8*	9.8±0.6	*34.6±4.8**
Prednisone propionate (A)	0.207	10	95.0± 8.5	259.0±25.0	11.9±0.4	43.9±3.3*
Prednisone propionate (B)	0.207	10	*51.4± 6.6*	198.2±15.7*	*10.4±0.3*	34.8±3.5*
Prednisone oenanthate (A)	0.235	18	111.0± 6.4	256.2±14.6	10.9±0.4*	48.2±3.4
Prednisone oenanthate (B)	0.235	20	*65.7± 6.4**	235.0±10.3*	10.9±0.4	45.2±3.0
Prednisolone (A)	0.180	10	81.0± 7.0	274.0±10.6	12.3±0.5	36.5±4.3
Prednisolone (B)	0.180	10	72.6± 9.2	254.2±15.5	*10.2±0.7*	30.7±2.0
Prednisolone oenanthate (A)	0.236	10	106.8±11.6	291.0±23.5	11.0±0.5	43.0±3.6
Prednisolone oenanthate (B)	0.236	10	81.4±11.0	259.7±13.9	11.0±0.3	36.0±3.9

(A) Daily dose in 0.5 cc. aqueous suspending vehicle.
(B) Daily dose dissolved in 0.2 cc. sesame oil.
† Mean values and standard errors are presented. When the values are significantly different ($P < 0.05$) from appropriate controls, they are printed in italics (steroids in oily solution versus aqueous suspension) or marked by asterisk (esterified steroids versus free ones).

The effectiveness of orally administered compounds seems to be increased by esterification with short chain fatty acids.

Experiments were also performed to investigate the possible role of the amount of the lipidic solvent on the specific activity of orally administered steroid hormones. The influence of the addition of bile acids, surface active agents such as polyoxyethylene sorbitan monooleate (Tween 80) and the replacement of the sesame oil with free fatty acids were investigated. Within a certain range, the amount of the lipidic solvent did not influence the specific activity of either the androgen or the cortical hormones. The addition of bile acids or surface active agents, or the replacement of sesame oil with oleic acid, have little influence on the specific activity of steroid hormones.

DISCUSSION

Although no direct evidence can be drawn from our data to explain the increased activity of the tested compounds when given orally in oily solution when compared to aqueous suspensions similarly administered the following hypotheses may be advanced:

1) Although it is generally assumed that the route of absorption of steroid hormones given orally is the portal vein, it may be supposed that, when dissolved in oil, a certain amount of ingested material reaches the blood stream by way of the lymphatic route, thus avoiding inactivation by the liver. This hypothesis does not agree, however, with the findings of Bloklage *et al.* (10) and Hyde *et al.* (19) who showed that the lymph collected from the thoracic duct in the rat contains only a small amount of steroid even when these were administered orally in oily solution.

2) Steroids in oily solution may be absorbed more slowly by the intestinal tract and therefore they may be present in the blood stream for a longer period of time than when they are administered in aqueous suspension. The administration in oil would effect the same condition as is obtained after repeated administration of a crystalline steroid; it is well

known that the biological effect of the hormones is greater, total dosage being equal, when they are given repeatedly over a period of time.

3) As stated in some reports concerning cholesterol (see 1–4), the lipidic solvent may also increase the absorption of steroid hormones. Some doubts, however, have been advanced about the role of fats in increasing cholesterol absorption (20–21). Furthermore it is generally assumed that cholesterol and steroid hormones are absorbed by different routes, *viz.* the former by the lymphatic route, the latter by the portal route. On the other hand, the hypothesis that the increased biological activity of the steroids dissolved in oil depends upon a higher degree of absorption obviously supposes that the same steroids are absorbed only in part from the aqueous suspension, which seems rather doubtful. Unfortunately, the amount of enteric absorption of steroids in the rat (9–14) has been largely evaluated by dissolving the substances in 50% ethyl alcohol, which may have facilitated a practically complete absorption of the administered dose.

4) Finally it may be supposed that the oil solvent protects the steroids from eventual transformations during the digestion and absorption processes.

As far as the administration of steroids in esterified form is concerned, our findings have shown that esterification with short chain fatty acids increased the biological activity of some compounds, i.e. dihydrotestosterone and prednisone.[1] This fact is already well known for steroids administered subcutaneously in oily solution. It is generally believed that esterified steroids achieve this greater activity by being more slowly absorbed from the injection site; whether such an explanation may also be applied to oral administration, we do not know. However, in the dog, Collins *et al.* (22) have found that the disappearance of plasma 17-OHCS following oral administration of some hydrocortisone esters at high dosage levels was considerably slower than after administration of free steroid. A more prolonged plasma steroid elevation following administration of hydrocortisone cyclopentylpropionate than after free hydrocortisone was also observed by Kelley *et al.* (23) in human subjects. If these results should be taken to indicate a slower rate of absorption of steroid esters than that of free ones (perhaps for the time involved in hydrolysis of the esters prior to absorption), then the explanation of our findings would be apparent. As however the methods used by the above mentioned authors did not rule out the possibility that the esters are absorbed in the intact form, then taken up and hydrolysed by the liver and so released more slowly than free steroids, the question cannot be clearly settled.

The finding that the activity of the dihydrotestosterone formate ester

[1] On this subject some unpublished observations have been quoted in the literature: in the rat oral administration of certain hydrocortisone esters resulted in an enhanced liver glycogen deposition activity (22), whereas in the dog hydrocortisone acetate, in doses up to 6 mg. per kg., did not elicit a physiological response (23). However, details of these studies are not available.

is high is worthy of consideration, because these esters have received little attention since the report of Miescher *et al.* (24) who demonstrated the activity of parenterally administered testosterone formate.

An interesting point brought out by our data is the lesser degree of androgenic activity (the myogenic activity being equal) exhibited by androstanolone and its esters and by testosterone esters when administered by the oral route when compared to subcutaneous administration. The difference is more evident when aqueous suspensions are employed.

More questions have been raised than answered by our work and further efforts will be necessary in order to clarify them.

REFERENCES

1. Kritchevsky, D.: *Cholesterol.* Wiley & Sons, New York. 1958.
2. Gould, R. G. and R. P. Cook: in *Cholesterol: Chemistry, Biochemistry and Pathology.* R. P. Cook (Editor). Academic Press, New York. 1958.
3. Glover, J. and R. A. Morton: *Brit. Med. Bull.* **14:** 226. 1958.
4. Favarger, P.: *Bull. Soc. Chim. Biol.* **40:** 1023. 1958.
5. Girard, A., G. Sandulesco and A. Fridenson: *C. R. Soc. Biol.* **112:** 964. 1933.
6. Emmens, C. W.: *J. Endocrinol.* **1:** 142 and 373. 1939.
7. Selye, H.: *Endocrinology* **32:** 279. 1943.
8. Hoffman, M. M., G. Masson and M. L. Desbarat: *Endocrinology* **42:** 279. 1948.
9. Ashmore, J., W. H. Elliott, E. A. Doisy, Jr. and E. A. Doisy: *J. Biol. Chem.* **200:** 661. 1953.
10. Bloklage, B. C., H. J. Nicholas, E. A. Doisy, Jr., W. H. Elliott, S. A. Thayer and E. A. Doisy: *J. Biol. Chem.* **202:** 27. 1953.
11. Hyde, P. M., W. H. Elliott, E. A. Doisy, Jr. and E. A. Doisy: *J. Biol. Chem.* **207:** 287. 1954.
12. Shen, N. C., W. H. Elliott, E. A. Doisy, Jr. and E. A. Doisy: *J. Biol. Chem.* **208:** 133. 1954.
13. Hyde, P. M., W. H. Elliott, E. A. Doisy, Jr. and E. A. Doisy: *J. Biol. Chem.* **208:** 521. 1954.
14. Bloklage, B. C., E. A. Doisy, Jr., W. H. Elliott and E. A. Doisy: *J. Biol. Chem.* **212:** 935. 1955.
15. Wells, J. A., M. W. Burrill and R. R. Greene: *Proc. Soc. Exper. Biol. & Med.* **45:** 452. 1940.
16. Alibrandi, A.: *Atti Reale Accademia Peloritana.* **46:** 5. 1946.
17. Hershberger, L. G., E. G. Shipley and R. K. Meyer: *Proc. Soc. Exper. Biol. & Med.* **83:** 175. 1953.
18. Cresseri, A. and A. Meli: *Arch. Sci. Biol.* **37:** 551. 1953.
19. Hyde, P. M., E. A. Doisy, Jr., W. H. Elliott and E. A. Doisy: *J. Biol. Chem.* **209:** 257. 1954.
20. Bollman, J. L. and E. V. Flock: *Am. J. Physiol.* **164:** 480. 1951.
21. Blomstrand, R. and E. H. Ahrens, Jr.: *J. Biol. Chem.* **233:** 327. 1958.
22. Collins, E. J., A. A. Forist and E. B. Nadolski: *Proc. Soc. Exper. Biol. & Med.* **97:** 558. 1958.
23. Kelley, V. C., A. K. Done, L. E. Ainger, R. S. Ely, A. A. Forist and E. J. Collins: *J. Pediat.* **49:** 26. 1956.
24. Miescher, K., A. Wettstein and E. Tschopp: *Biochem. J.* **30:** 1977. 1936.

VASCULAR RESPONSES IN THE PITUITARY STALK[1]

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ABSTRACT

The superior hypophyseal arteries, primary capillaries, and portal trunks of the hypophyseal portal system were studied in more than 150 living albino mice following a standardized operative procedure.

Direction of blood flow, vasomotor activity, and changes in linear velocity of blood flow in portal trunks were studied following the administration of several different drugs, the application of several different physical stimuli, and the manipulation of the arterial blood supply and venous return from the brain case.

Evidence is presented for the validity of linear velocity in the portal trunks as a rough indicator of total blood flow into the anterior pituitary. The probability that vasomotor activity and variations in portal blood flow affect anterior pituitary function is discussed. The effects on the superior hypophyseal arteries of adrenergic and cholinergic drugs are described, and the relationships of vascular phenomena to the susceptibility of the anterior pituitary to infarction are considered.

IN THE almost 30 years since its description by Popa and Fielding (1), the hypophyseal portal system of vessels of the pituitary stalk has come to assume an ever more important place in the thinking of endocrine physiologists. The hypothesis of humoral control of the anterior pituitary by the hypothalamus, mediated directly by way of the portal vessels, has been developed by Harris, Green and others, and has the accumulated evidence derived from the experiments of many workers to support it. The subject has been reviewed in several recent papers (2, 3, 4, 5). In conjunction with the extensive physiologic experimentation which has brought the neurohumoral hypothesis into its present position of strength, a number of morphologic studies on the hypophyseal portal system of vessels have been carried out in various species of animals. These have been made to determine the fine ramifications of its vascular structure and to determine the direction of blood flow in the portal vessels. Although the concept of the flow of blood in the portal vessels from the anterior pituitary toward the hypothalamus no longer seems to have very much support, a brief review of the literature bearing on this point seems pertinent to the data given below.

Despite some comparatively recent support by Spanner (6), Schmidt (7), and others for Popa and Fielding's concept that portal blood flows

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from pituitary to the hypothalamus, fairly strong evidence that this concept was in error appeared quite early. In 1936, on the basis of purely morphologic studies in Rhesus monkeys, Wislocki and King (8) concluded that the direction of blood flow in this portal system of vessels must be downward from the median eminence into the pars distalis; and, in 1935, Houssay, Biasotti, and Sammartino described this direction of flow in the living toad (9). Studies on dead tissue preparations in a number of different species of animals [including a long comparative study by Green (10), studies in the rat, rabbit and dog by Green and Harris (11), in the cat by Morato (12), in the rat, mouse, guinea pig and bat by Morin and Bottner (13), in the rat by Landsmeer (14), in the human by McConnell (15) and by Xuereb, Prichard, and Daniel (16, 17), and in the sheep, rat and goat by Prichard and Daniel (18, 19, 20, 21)] have all supported this concept of hypothalamic-hypophyseal flow. In 1956, Spatz, earlier a proponent of the hypophyseal-hypothalamic direction of flow, modified his view on this question in the light of new evidence (22).

The studies using techniques involving direct visualization of hypophyseal portal vessels in the living animal have thus far all supported the concept of blood flow from the hypothalamus downward into the anterior pituitary. These studies include the original investigation of Houssay, *et al.* in the toad; Green, in the bullfrog (23); Green and Harris, in the rat (24); Barnett and Greep, in the rat (25); and Worthington, in the mouse (26), rat and frog (27).

Despite the large number of experimental studies which have been made to test a concept (the neurohumoral hypothesis) which involves directly the hypophyseal portal system of vessels, and despite the many careful anatomical studies which have been made of this system, attempts to obtain data relating to its physiology have been limited to those few studies in living animals cited above.

Information about the physiology of the hypophyseal portal system of vessels seems important. Such information should contribute to a more complete understanding of the functioning of the normal gland. It should also contribute to a more complete understanding of the functioning of the gland under the various experimental conditions which have been imposed upon it. Since technical problems all but eliminate the use of classical physiological methods, direct microscopic observation in living animals was undertaken.

MATERIALS AND METHODS

The experiments and observations described in this paper were made in over 150 albino mice of the Carworth Farms strain (CF_1). The majority of the animals were male and their weight ranged from 16 gm. to 30 gm. Animals which had just reached maturity were most frequently used, and all mature animals which were used grew to maturity in the environment of the laboratory. Each animal was checked prior to use for color and sleekness of coat, texture of skin, state of hydration, and weight.

Animals were anesthetized with intraperitoneal injections of sodium pentobarbital. The initial dose was .01 cc./gm. of body weight of a 6.5 mg./cc. solution. This dose was supplemented as often as necessary and in sufficient amounts to maintain the desired level of anesthesia. The depth of anesthesia was defined as follows:

(1) Light. Making spontaneous movements or responding with movement when touched. Rapid respiration.

(2) Moderate. Responding with movement only to a mildly painful stimulus. Slower respiration.

(3) Deep. Not responding with movement to any stimulus. Respiration slow and shallow.

With practice each of these levels of anesthesia could be defined within limits by inspection and by touching without resorting even to a mildly painful stimulus.

Sodium pentobarbital was found to be the most satisfactory agent of the several anesthetics which were tested. The margin of safety was greater, the depth of anesthesia could be better controlled, and this drug seemed to interfere less with vascular reactions in the stalk blood vessels. Administration of this drug was, however, associated with some vascular effects, and these are described with the other observations.

The superior hypophyseal arteries and arterioles, the primary capillaries and the hypophyseal portal vessels were exposed by the technique described by Worthington (26). This technique consists of approaching the under surface of the brain through a ventral midline incision. The trachea is divided after removal of the muscles overlying it, and the cut lower end is supported at the sternal notch. The upper end is retracted anteriorly. The esophagus is divided and the ends are allowed to retract. The attachment of the exposed longus capitis muscle is scraped away, and the outer table of bone is removed from a point just posterior to the synchondrosis of the basisphenoid to a point just posterior to the transverse sinus. The use of bone wax prevents bleeding from bone. If wide anterior exposure is desired, the transverse sinus may be plugged with bone wax. The inner table of the bone is removed by insinuating a fine dental chisel through the synchondrosis of the basisphenoid and snapping out the remaining thin plate of bone. The exposed area may be irrigated as often as necessary with mammalian Ringer's solution, and the excess removed by a fine-tipped suction tube. Details of structure beneath the dura may be observed without removing this transparent membrane; however, the area may be studied in greater detail if the dura is removed. This is an optional procedure and entails much additional risk of local damage.

Animals showing signs of local damage in the region of study were discarded as were those in which there had been excessive blood loss during the procedure. The single exception to this was that a number of animals whose only abnormality had been loss of blood during the operation were studied with care in order to define some of the effects of blood loss on hypophyseal portal blood flow.

Observations were made with a binocular monobjective compound microscope, equipped with a 10× objective and 10, 12½, and 15× oculars, and with a Leitz binocular biobjective dissecting microscope using 12½× oculars and 4, 6, 8, and 12× objectives. Higher magnifications have not been obtained because of technical difficulties. The exposed field was illuminated by a beam of light directed obliquely onto the field. Two heat-absorbent glass filters were placed in the beam. The surface of the objective was treated with Aerosol to prevent fogging.

The epinephrine used in these experiments was l-epinephrine bitartrate in the form commercially available as "Suprarenin" from Winthrop-Stern. The acetyl-beta-methylcholine was in the form of Mecholyl chloride obtained from Merck and Company. The nor-epinephrine was l-nor-epinephrine bitartrate obtained from Winthrop-Stern. The morphine was in the form of morphine sulphate hypodermic tablets dissolved in water.

OBSERVATIONS

General observations. The pituitary stalk of the living mouse and some intial observations on vascular reactions of the vessels of the hypophyseal portal system were described in an earlier paper (26). The reactions to drugs and stimuli described below are those which may be induced following the operative procedure described in that paper and were observed in animals under light to moderate sodium pentobarbital anesthesia.

In the animals described in the earlier paper, blood flow in all of the hypophyseal portal trunks of the stalk was in each case downward into the pars distalis. Reversals in direction of flow were observed only in the primary capillaries and arterial anastomoses around the stalk. These observations have been confirmed in all of the animals of this longer second series, not only in those subjected to anesthesia and operation only, but also in those subjected to the variety of drugs and stimuli described below.

The arterial vessels supplying the primary capillary system are highly contractile, and varying numbers of primary capillaries may be lost to view for varying periods. However, active contractility of the hypophyseal portal trunks has never been observed. Furthermore, the study of serially-sectioned mouse pituitary stalks has supplied no evidence which indicates the presence of contractile elements in the walls of the hypophyseal portal vessels. Although it cannot be stated positively that they cannot contract, the number of animals studied and the variety of stimuli involved make it seem most unlikely that they do. Narrowing of the diameter of the column of red cells in the portal vessels has been observed only in animals subjected to sudden severe respiratory and vascular embarrassment due to deliberate overdosage with nor-epinephrine. Even in these circumstances there was no detectable change in the diameter of the lumen of the vessel. Change in the diameter of the vessel itself has been observed only when there was some passive distention following injections of large doses of sympathomimetic drugs. This change is presumably due to marked elevation of the systemic blood pressure.

It becomes probable then that change in linear velocity of the blood flow in the hypophyseal portal vessels is due, when it occurs, not to any independent contractility of the portal trunks themselves, but to combinations of factors lying proximal in the circulatory system to the portal trunks. Such change in the linear velocity in the portal trunks has been observed many times in conjunction with many different stimuli.

Effects of sodium pentobarbital. The first of the drugs which requires consideration is sodium pentobarbital. Since it has been used for anesthetizing all of the animals in this series, its effects had to be carefully studied before other drugs could be tested.

Under light sodium pentobarbital anesthesia, portal blood flow is quite rapid. If a moderately heavy supplementary dose of the drug is given intraperitoneally, within 5 minutes the rate of portal blood flow is diminished

and the arteries and arterioles become dilated. After 15 to 20 minutes, no difference in the rate of blood flow from that observed before the administration of the supplementary dose can be seen, and the arterial vessels have narrowed somewhat. Heavy sublethal doses of sodium pentobarbital give a similar, but more marked and prolonged effect, and more uniform and prolonged anesthesia. When a smaller initial dose of the anesthetic is used with more frequent supplementation, more clear-cut responses of the hypophyseal vessels are seen than when an initial heavy dose is employed. The administration of the supplementary doses of sodium pentobarbital is associated with a decrease in the rate of respiration.

Because of these effects, the time of administration of additional drugs has been related to the time of administration of the last preceding dose of supplementary anesthetic, and great care has been used in interpreting observations.

Effects of morphine. Morphine sulphate, a blocking agent for the adrenocorticotrophic response to stress (28), has been tested for its effects on portal blood flow and the vessels of the pituitary stalk. In animals under very light sodium pentobarbital anesthesia, the intraperitoneal injection of 2 mg. to 3 mg. of this drug per 100 gm. of body weight is followed by a reduction of portal flow rate. (These animals were so lightly anesthetized that they were spontaneously pulling at their restraints.) After the changes initially associated with an injection of sodium pentobarbital supplement can no longer be detected, morphine causes a marked reduction in portal flow rates and dilatation of both hypophyseal arteries and arterioles. In addition, as might be expected, morphine seems to interfere with the response of the hypophyseal arterioles and portal blood flow to painful stimuli. The reaction to morphine of reduction of portal blood flow and dilatation of the arterial vessels seems to be in all respects similar to that caused by sodium pentobarbital alone.

Effects of epinephrine. The effects of epinephrine applied locally to the pituitary stalk in the mouse have been reported previously (26). In dilutions as great as 1:5,000,000 the effects of such local application are arterial constriction and reduction in the rate of portal blood flow. Following the administration of l-epinephrine, a single arteriole and a single group of primary capillaries have sometimes been observed to disappear from the field of study temporarily.

A single dose of epinephrine given subcutaneously or intravenously (2 to 5 μg. per kg.) is followed by the gradual acceleration of portal flow rates, and a minimal transient narrowing of the arterial and arteriolar vessels. This is followed by return to their original, or slightly larger, diameters. The phase of arterial constriction lasts for about one minute. If an overwhelming sublethal dose of epinephrine is given intravenously, there is transient spasmodic contraction of all of the arterial vessels, followed by more prolonged arterial constriction and portal acceleration. As pointed

out in a previous paragraph, extremely high but sublethal does of epinephrine given in this manner are capable of distending the portal trunks and result in engorgement of all the vessels of the stalk, including the primary capillaries.

Effects of nor-epinephrine. Local and peripheral administration of L-nor-epinephrine bitartrate has given inconsistent results. In some experiments low concentrations of nor-epinephrine were extremely active when applied locally, in that they resulted in profound arteriolar constriction and reduction in portal flow rates. In other experiments the effects of this drug applied locally were minimal, with very slight narrowing of arterial vessels, and a considerably more moderate effect on the rate of portal blood flow.

In some cases when the drug was given in high dilutions as a very slow intravenous infusion, there occurred an intense arteriolar constriction around the median eminence and stalk and reduction in portal flow rates. In other experiments the results of the peripheral administration of nor-epinephrine were essentially similar to those observed with the peripheral administration of epinephrine and the drug had to be given in at least the same dose.

Large unphysiological but sublethal doses of nor-epinephrine given intravenously have, however, given more consistent results. Such a large dose resulted in respiratory embarrassment, cessation of blood flow in the portal trunks and primary capillaries and temporary cessation or extreme slowing of the rate of blood flow in the superior hypophyseal arteries and arterioles. This is followed by intense arteriolar constriction and extremely rapid portal blood flow.

Effects of Mecholyl. The local application of Mecholyl in concentrations of 1:1,000 to 1:100,000 has consistently resulted in arterial and arteriolar dilatation and the acceleration of portal blood flow. This observation was made in an earlier series, and all subsequent observations agree.

Effects of physical stimuli. Certain stimuli were applied for the purpose of discovering whether or not the caliber of the vessels of the stalk and the rate of blood flow in the portal trunks could be detectably altered by such stimuli. In the animal under moderate anesthesia, the application of a painful stimulus has been observed to induce arterial and, more particularly, arteriolar vasoconstriction around the median eminence and stalk and is associated with acceleration of hypophyseal portal blood flow. Such a stimulus must be intense, however. A response may be elicited on occasion by such stimuli as pinching the tail or toe, but with this stimulus, changes in the rate of blood flow in the portal vessels usually cannot be detected. However, a drop of hypertonic saline injected under the skin or the application of a thermal burn to back or extremities almost always results in distinct acceleration. The sequence of events under these conditions is as follows: Subcutaneous injection of hypertonic saline is immediately followed by constriction of the arterioles, then spurts of acceleration and slowing in the hypophyseal portal vessels, followed by a persistent acceleration. Applica-

tion of thermal burn results in an intense arterial and arteriolar vasoconstriction, a temporary slowing of the rate of blood flow in the portal vessels, followed by marked acceleration. These changes are associated with an increase in the rate of respiration.

The total temporary occlusion of the tracheotomy opening, or more prolonged partial occlusion of the tracheotomy opening, results in the widest dilatation of the arterial vessels which has been seen following the application of any drug or stimulus, and at the same time there has been marked acceleration of portal blood flow.

While a painful and traumatic stimulus in the form of a burn applied to the back or to the extermities results in intense constriction of arteries and arterioles around the pituitary stalk and a marked acceleration in the rate of portal blood flow, the same type of burn stimulus applied to the very thin anterior abdominal wall results in arterial dilatation. The result was sometimes obtained by injuring rather small portions of tissue such as the thin edge of the liver or portion of the gastrointestinal tract.

Hemorrhage was induced by cutting one of three vessels: a small, constant superficial vessel in the groin, the inferior vena cava, or the renal vein. Cutting of the small vessel in the groin is attended by hemorrhage of approximately .2 cc. This hemorrhage is usually self-limited. At its onset, the rate of blood flow in the portal vessels slows for a short period (about 30 sec.). This is followed by acceleration. Sudden large hemorrhages, such as occur when the vena cava is cut (not ending with death in a few minutes), are followed by an almost immediate, marked reduction in the rate of portal blood flow and an arterial dilatation. The reduction in portal rates of flow and the arterial dilatation are usually prolonged and seldom do these conditions correct themselves before the experiment is terminated. Sudden large hemorrhage is not necessary for bringing about this response. Animals which have bled intermittently and in small amounts from vessels in the bone during the operative procedure have frequently shown arterial dilatation and an overall rate of portal flow which was considerably less than that in an animal which has not experienced such hemorrhages.

An almost completely uncontrolled hemorrhage, such as occurs when the renal vein is cut, results in death in about 10 minutes. There is wide arterial dilatation, marked and progressive reduction of the rate of portal blood flow and, finally, cessation of portal flow at the time of death. However, when the lethal agent is hemorrhage alone, the hypophyseal portal circulation fails only just before the arterial circulation and usually after respiration has ceased.

Cessation of portal blood flow in the portal vessels of the upper portion of the stalk may be induced by interrupting the superior hypophyseal arteries. In some cases of unsatisfactorily prepared animals in which pressure was inadvertently exerted on the lower reaches of the portal trunks as they fan out into the pars distalis, reduction in the rate of blood flow and

thrombosis occurred. Bilateral common carotid ligation results in a very marked slowing of the rate of blood flow in the portal vessels and the primary capillaries but does not result in cessation of blood flow in these vessels. Apparently, there is a sufficient vertebral circulation to support continued circulation within the portal system.

DISCUSSION

The rather severe limitations of this type of preparation and problems of interpretation associated with the observations described have been discussed in a previous paper (26). To review this point briefly, the animal has been subjected to anesthesia and to a very traumatic operative procedure. It may well be in a state of maximal stress or may have passed through such a state. There is no assurance that the vascular activity observed in such an animal is anything like that in a normal unanesthetized intact animal. Most, but not all, of the large number of capillaries in the pituitary stalk have been patent at least some of the time and usually all of the time in almost every animal which has been examined. However, it is apparent from the data presented above that, even under these extreme conditions, the vessels of the pituitary stalk do show some variation and some response to a number of drugs and peripheral stimuli.

These observational data are presented as a preliminary attack on the problem of the relation of hemodynamic factors to neurohumoral mechanisms and the control of the anterior pituitary. The possibility of such a relationship has been suggested by a number of different workers (29, 30, 31, 32, 5 and others). However, the emphasis on specific neurohumors has tended to divert attention from an area which might well supply important and even essential data for a more complete understanding of anterior lobe function.

Since linear velocity of portal blood flow is being used as a rough indicator of total anterior lobe blood flow in this discussion, the following comments are made in support of the validity of this indicator.

First, under control and all experimental conditions thus far tested there is no active contraction of the wall of the hypophyseal portal vessels. Therefore, in all probability there will be no changes in linear velocity which are dependent solely on vasoconstriction in portal trunks.

Second, there is evidence, derived from sheep, rat, goat, and man, that the portal vessels of the stalk are the only blood supply to the anterior lobe (15–21). The only recognized exception is the rabbit (33).

Third, sections of the pituitary stalk of the mouse reveal no major structural differences between those portal trunks on the surface and those which are deeper. In the leopard frog, Rana pipiens, in which the entire stalk can be transilluminated, there seems to be no essential difference between the structure and behavior of those vessels which are closer to the surface and those which are deeper.

Fourth, the essential point has been made repeatedly that the observed direction of flow in the portal vessels is downward from the hypothalamus to the pituitary. The animals reported in this paper, taken together with previous data on 27 rats (24, 27), 50 mice (26), and 9 cats (34) give confirmation of this point in over 236 mammals of three different species. This is in addition to the work on living amphibia previously referred to.

Thus, in all probability, we have a non-contractile set of portal trunks which conducts blood from the median eminence to the pars distalis and which, in many animals, serves as the sole source of metabolites for the pars distalis. It is likely, therefore, that observable portal rates of flow are a valid rough indicator of the rates at which substances in the general circulation and, more specifically, in primary capillary blood may be supplied to the pars distalis.

Although experiments have shown that small percentages of anterior pituitary tissue in the sella turcica will support adequate anterior pituitary function (35), it does not necessarily follow that such experiments have validity when applied to the question of the definition of an adequate blood supply to the anterior pituitary. It seems to be quite possible that a fairly small percentage of anterior pituitary tissue adequately supplied with portal blood might be considerably better able to support normal anterior pituitary function than the entire gland morphologically intact but with the rate of blood supply reduced to less than the minimum required for the formation, release, and carrying away of any given trophic hormone.

Campbell and Harris (36) have shown that in stalk-sectioned rabbits the pars distalis atrophies to about an equal degree, whether or not a paper plate is inserted between the cut ends of the stalk, and that those without plates show greater functional activity. Since there is approximately equal atrophy and since there are approximately equal numbers of India ink-filled capillaries present, they conclude that atrophy due to a decreased blood supply is not a factor in decreased function. It is apparent from their data, however, that the presence of the plate must have reduced the number of afferent vascular channels into the anterior lobe. For this reason, there remains the possibility that the presence of the plate may be sufficient to reduce the rate of blood supply below the critical level for adequate function without causing outright infarction, and that this may be a contributing factor in the reduction in functional activity observed. The presence of an artery below the level of the sella diaphragm in the rabbit does not detract from this possibility.

There are several points of interest relating to the action of drugs and stimuli on the rate of blood flow in the portal trunks.

Two "blocking agents" (Nembutal and morphine) bring about a distinct reduction in the rate of the hypophyseal blood flow. Even small supplementary doses of sodium pentobarbital in an animal which is under very light anesthesia will bring about a distinct slowing of hypophyseal portal

blood flow and arterial dilatation, and heavy but sublethal doses of the same drug will bring about a quite marked slowing of the rate of blood flow. In the case of morphine, it may be protested that the drug was tested in an animal under sodium pentobarbital anesthesia. However, in an animal so lightly anesthetized with sodium pentobarbital that it was pulling at the restraints and was probably conscious, the administration of morphine resulted in slowing. It seems possible that these drugs (and perhaps others) which are known to block the release of certain anterior pituitary hormones are acting in part through reduction of the rate of blood flow into the gland.

Conversely, the peripheral administration of epinephrine, the application of a painful stimulus, the obstruction of the animal's air supply or the application of a stimulus both painful and traumatic (such as thermal burn to the extremities or back) results in a rate of blood flow well above that observed in the control animal. These types of stress stimuli are known to bring about the release of adrenocorticotrophic hormone (37). Here again it might be hypothesized that part of the effects of these stimuli was not being mediated entirely by activation of the nervous system, but by increasing the rate at which materials necessary for the production or release of specific hormones of the anterior pituitary were being supplied to the gland.

What vascular factors are necessary for normal functioning of the anterior pituitary? Are specific changes in the vascular apparatus sufficient in themselves to activate the anterior pituitary? It is reasonable to assume that an intact and normally functioning vasculature is necessary for the normal functioning of the gland. It is quite difficult, however, to define a normally functioning vascular apparatus for the pituitary. The mere presence of injectable capillaries in any number is not sufficient evidence that the anterior pituitary (or any other tissue) in the living state is receiving blood at a sufficient rate to maintain any given function. It is possible that sufficient blood may be passing through the sinusoids of the anterior pituitary to maintain the viability of the cells of the gland parenchyma but at a rate insufficient to maintain one or more of the functions of the glandular cells of the organ. Since two of the blocking agents which have been shown to interfere with functions of the anteroir pituitary are also capable of markedly reducing the rate of blood flow through the gland, the possibility that they are acting in part through vascular effects should be recognized until there is evidence to exclude it.

Whether or not vascular changes alone are sufficient to stimulate any of the secretory activities of the anterior pituitary, the data given in this paper do show that, in some circumstances usually associated with the release of adrenocorticotrophic hormone, there is an unusual increase in the rate of blood flow through the gland. Rapid delivery of pituitary hormones to target organs might well necessitate rapid blood flow through pituitary tissue. Until it has been clearly demonstrated that increases in rate of blood

flow are unnecessary for increased delivery of hormones to one or more target organs, the possibility should not be ignored.

The preceding comments, which have been directed toward changes in the rate of supply of substances in the general circulation, may be applied with equal force to specific neurohumors delivered into the portal blood. The neurohumoral hypothesis, as previously noted, is supported by a mass of indirect evidence and apparently awaits only the isolation and demonstration of the substance or substances involved, for final proof. The striking experiments of Nikitovitch-Winer and Everett (38) have demonstrated quite clearly that transplanted anterior pituitary tissue must be placed in close relationship to the median eminence and its quite specialized set of vessels for normal gonadotrophic function to be maintained. If the neurohumoral hypothesis is correct, increase in rate of portal blood flow may be acting as a facilitating mechanism in the formation and/or release of adrenocorticotrophic hormone, luteinizing hormone and follicle-stimulating hormone.

Luteotrophic hormone is a special case. Evidence of increased luteotrophic hormone secretion from pituitary transplants away from the median eminence (39), induction of lactation and pseudopregnancy (40, 41) with drugs which depress the central nervous system, and lactation following stalk section (42) all imply inhibitory action by the hypothalamus. Since all of these procedures involve interference with the portal blood supply, or the probability thereof, and since variations in rate of portal flow have been amply demonstrated, the possibility of a degree of control of LTH secretion, mediated by the rate at which a specific inhibitory substance might be supplied to the gland, must be considered. In the case of transplants away from the median eminence, this rate would approach zero. Still another possibility is a reduction in the rate of supply of some less specific inhibitory material in the general circulation. This latter concept would eliminate necessity for hypothesizing a specific inhibitory hypothalamic neurohumor for LTH.

In summary, a variety of stimuli alter markedly the rate of hypophyseal blood flow. The possibility that such alterations are related to alterations in functional activity, which may or may not be related to specific neurohumors, has not been excluded, and what constitutes an adequate blood supply to the anterior pituitary has not been satisfactorily defined.

Observations on the effects of hemorrhage are of interest from the point of view of the potential effects of shock on anterior pituitary blood supply and function. The occurrence of anterior lobe infarction following postpartum hemorrhage (43), the studies of Prichard and Daniel showing localized infarction in the anterior lobe following the cauterization of individual portal trunks in the rat (20), the presence of fine, highly contractile arterioles (26), and the portal trunks and two sets of capillaries interposed between the cerebral arteries and the venous sinuses of the brain case, suggest the

possibility of a relatively high susceptibility to factors leading to vascular insufficiency. However, in these experiments, in the case of hemorrhage alone, portal blood flow never failed completely until just before death. Furthermore, attempts to induce a sufficient degree of hemorrhagic shock to produce thrombosis of the portal vessels met with failure. In addition, bilateral occlusion of the common carotids, while interfering markedly with the rate of blood flow in the hypophyseal portal vessels, was not sufficient to eliminate flow completely. On the other hand, in three cases in which adrenalectomized animals were examined two to five days following adrenalectomy (27), gradual reduction in the rate of portal blood flow and stasis occurred 45 minutes to 1 hour before death ensued. Thus, it would seem that factors other than those associated with acutely induced hemorrhagic shock alone are necessary to produce thrombosis in the portal vessels.

Preliminary experiments have been made on the effects of occluding portions of the venous drainage of the head and increasing intracranial pressure. The results suggest that occlusion of venous outflow and pressure on veins within the brain case are more effective in reducing the rate of hypophyseal portal flow than is hemorrhagic shock or partial interference with the arterial supply.

The experiments with epinephrine and nor-epinephrine acting as local agents indicate that each of these drugs is a constrictor when applied locally to the superior hypophyseal vessels. The results observed with Mecholyl suggest that cholinergic drugs of this type are vasodilators of pituitary arterial vessels when acting locally. These results, taken with the responses to some of the physical stimuli, indicate the presence of autonomic vasodilator and vasoconstrictor mechanisms for the superior hypophyseal arteries.

REFERENCES

1. Popa, G. T. and U. Fielding: *J. Anat.* **65:** 88. 1930.
2. Benoit, J. and I. Assenmacher: *J. physiol.*, Paris **47:** 427. 1955.
3. Donavan, B. T. and G. W. Harris: *Ann. Rev. Physiol.* **19:** 439. 1957.
4. Sawyer, C. H. and B. V. Critchlow: *Ann. Rev. Physiol.* **19:** 467. 1957.
5. Sayers, G., E. S. Redgate and P. C. Royce: *Ann. Rev. Physiol.* **20:** 243. 1958.
6. Spanner, R.: *Klin. Wchnschr.* **30:** 721. 1952.
7. Schmidt, C. G.: *Frankfurt. Ztschr. Path.* **63:** 172. 1952.
8. Wislocki, G. B. and L. S. King: *Am. J. Anat.* **58:** 421. 1936.
9. Houssay, B. A., A. Biasotti and R. Sammartino: *Compt. rend. Soc. biol.* **120:** 725. 1935.
10. Green, J. D.: *Am. J. Anat.* **88:** 225. 1951.
11. Green, J. D. and G. W. Harris: *J. Endocrinol.* **5:** 136. 1947.
12. Morato, M. J.: *Anat. Rec.* **74:** 297. 1939.
13. Morin, F. and V. Bottner: *Morphol. Jahrb.* **85:** 470. 1941.
14. Landsmeer, J. M. F.: *Acta anat.* **12:** 82. 1951.
15. McConnell, E. M.: *Anat. Rec.* **115:** 175. 1953.
16. Xuereb, G. P., M. M. L. Prichard and P. M. Daniel: *Quart. J. Exper. Physiol.* **39:** 199. 1954.

17. Xuereb, G. P., M. M. L. Prichard, and P. M. Daniel: *Quart. J. Exper. Physiol.* **39:** 219. 1954.
18. Daniel, P. M. and M. M. L. Prichard: *Quart. J. Exper. Physiol.* **42:** 237. 1957.
19. Daniel, P. M. and M. M. L. Prichard: *Quart. J. Exper. Physiol.* **42:** 248. 1957.
20. Daniel, P. M. and M. M. L. Prichard: *Quart. J. Exper. Physiol.* **41:** 215. 1956.
21. Prichard, M. M. L. and P. M. Daniel: *Am. J. Path.* **34:** 433. 1958.
22. Spatz, H.: In Pathophysiologia Diencephalica, ed. by Curri, S. B., L. Martini, and W. Kovac, Springer-Verlag, Wien. pp. 53–77. 1958.
23. Green, J. D.: *Anat. Rec.* **99:** 21. 1947.
24. Green, J. D. and G. W. Harris: *J. Physiol.* **108:** 359. 1949.
25. Barrnett, R. J. and R. O. Greep: *Science* **113:** 185. 1951.
26. Worthington, W. C., Jr.: *Bull. Johns Hopkins Hosp.* **97:** 343. 1955.
27. Worthington, W. C., Jr.: Unpublished data.
28. Briggs, F. N. and P. L. Munson: *Endocrinology* **57:** 205. 1955.
29. Harris, G. W.: *Physiol. Rev.* **28:** 139. 1948.
30. Stutinsky, F., M. Bonvallet and P. Dell: *Ann. endocrinol.* **10:** 505. 1949.
31. Green, J. D.: *Am. J. Anat.* **88:** 225. 1951.
32. Barrnett, R. J. and R. O. Greep: *Endocrinology* **49:** 337. 1951.
33. Harris, G. W.: *J. Anat.* **81:** 343. 1947.
34. Barham, J.: Personal communication.
35. Ganong, W. F. and D. M. Hume: *Endocrinology* **59:** 293. 1956.
36. Campbell, H. J. and G. W. Harris: *J. Physiol.* **136:** 333. 1957.
37. Sayers, G. and M. A. Sayers: *Recent Progr. in Hormone Res.* **2:** 81. 1948.
38. Nikitovitch-Winer, M. and J. W. Everett: *Endocrinology* **63:** 916. 1958.
39. Nikitovitch-Winer, M. and J. W. Everett: *Endocrinology* **62:** 522. 1958.
40. Sawyer, C. H.: *Anat. Rec.* **127:** 362. 1957.
41. Barraclough, C. A.: *Anat. Rec.* **127:** 262. 1957.
42. Eckles, N. E., G. Ehni and A. Kirschbaum: *Anat. Rec.* **130:** 295. 1958.
43. Sheehan, H. L.: *J. Path. & Bact.* **45:** 189. 1937.

THE EFFECT OF THYROXINE ANALOGUES ON A TRANSPLANTABLE MOUSE PITUITARY TUMOR[1]

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ABSTRACT

Twenty-seven compounds structurally related to thyroxine were tested for their effect in depressing the growth of a TSH secreting pituitary tumor. After the transplanted tumor had grown to approximately 1.5 cubic centimeters in radiothyroidectomized hosts, compounds were injected daily for various periods of time. Measurements of tumor size were made weekly. Quantitative data were obtained relating amounts of the compounds required to suppress tumor growth. Activity of the analogues was calculated in terms of relative potency compared to L-thyroxine. Triiodothyronine was the only compound as effective as L-thyroxine. No compounds were found more effective. Most analogues compare well when compared on the basis of preventing a thiouracil induced goiter and effectiveness in inhibiting tumor growth. No compound which failed to show thyroxine-like activity, by previously reported methods, was found capable of preventing tumor growth. No compound even in high doses caused the completed disappearance of established tumors.

IN 1949 Gorbman (1) reported that the administration of radioactive iodine resulted in the development of pituitary tumors in some strains of mice approximately a year later. Furth and associates (2, 3, 4) later showed that such tumors could be transplanted into radiothyroidectomized mice and ultimately, after many transplantations, into normal hosts. These tumors produced large amounts of TSH which markedly stimulated the thyroid gland of normal mice bearing tumor transplants. It was also found that the administration of either thyroxine or thyroglobulin to tumor-bearing mice inhibited the growth of the pituitary tumor (5).

Recent studies have indicated that the biological activity of thyroxine analogues varies somewhat depending on the method used for study (6, 7, 8). The question arose as to whether some of the analogues which have shown little or no activity in previously reported studies might be active in inhibiting the growth of transplantable pituitary tumors. The present

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study was undertaken for this reason and with the hope of obtaining some insight into the factors involved in growth and function of the pituitary tumors.

MATERIALS AND METHODS

Most of the analogues were synethsized in the Warner-Lambert Research Institute.[3] The compounds were dissolved as previously described (6) and injected subcutaneously into mice daily in a volume of 0.1 ml. Fresh solutions were made at the start of each study and aliquots for daily use separated into small vials which were kept frozen until used. For quantitative studies each compound was tested at 3–6 different concentrations in groups of five mice bearing tumors of 1–2 cc.

The two original pituitary tumors used were obtained from Dr. Jacob Furth of Boston. Both were dependent for growth upon the absence of the thyroid and secreted, as far as we have determined, only the thyroid stimulating hormone. These tumors (LA3052A and LA3052B) came from the same mouse and had been carried through five generations as sublines by Dr. Furth. For quantitative evaluation of the biological activity of thyroxine analogues only mice bearing tumor LA3052A were used.

Approximately 600 LAF1 male mice about six weeks old when obtained from Jackson Laboratories were used. When the animals arrived they were placed on Remington low iodine diet for 2 weeks. At the end of this period all mice were given a single intraperitoneal injection of 55–65 μc. I^{131}. Three days later the animals were placed on regular laboratory chow.

Tumor tissue for transplantation was obtained from hosts bearing tumors between 1 and 2 cc. in size. Aseptic transplantation was done 2–3 weeks following the administration of I^{131}. Tissue was chopped finely and approximately 5 volumes of Eagle's solution added. Between 0.1–0.2 ml. of this suspension was injected intramuscularly into the right thigh using an 18 gauge needle.

The first sign of tumor growth was seen usually about 3 months after transplantation. Tumors were measured weekly by means of calipers in three different planes. Tumor volume was calculated by multiplying these values (in cm.) to give a volume in cc. The calculated value was found to be somewhat higher than the actual weight determined at autopsy. This discrepancy reflects mainly the irregularity of tumor shape and surrounding tissue, especially in animals bearing large tumors. For practical purposes this value proved useful since weekly growth curves of treated animals could be compared with controls without killing mice.

RESULTS

Experimental studies were started in animals bearing tumors 1–2 cc. in size. For the first 2–3 weeks tumor growth was not marked in control animals (Figs. 1 and 2). By 4 weeks the tumor mass had increased approximately 5–6 times in size. Thereafter the tumors grew rapidly until the death of the hosts. Treatment of tumor-bearing animals with active compounds depressed growth of tumors roughly in proportion to the dosage. This is illustrated in Figure 1 where 6 different doses of L-thyroxine are

[3] Smith, Kline and French Laboratories furnished the 3,5,3′-triiodo-L-thyronine. Travenol Laboratories, Inc. supplied the D-thyroxine and L-thyroxine. The 3,5,3′-triiodothyrobutyric acid and 3,5,3′,5′-tetraiodothyrobutyric acid were obtained from Cyclo Chemical Company. The 3,5,3′,5′-tetrachloro-D,L-thyronine was furnished by Dr. J. Lerman and synthesized by Sir C. R. Harington.

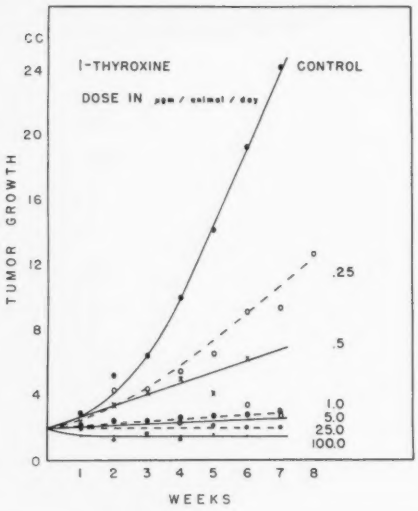


FIG. 1. The response of a transplantable pituitary tumor to 6 different doses of L-thyroxine compared with control animals.

compared. A dose of 1 microgram or more of L-thyroxine completely prevented tumors from growing, but doses 100 times greater failed to cause disappearance of established tumors. The higher doses of L-thyroxine did, however, cause weight loss and death of some animals.

Figure 2 shows a similar study done with D-thyroxine and illustrates that some doses (for example 4 µg. of D-thyroxine) slowed initial tumor growth for 4–5 weeks. After this time the tumors began to grow and, with some

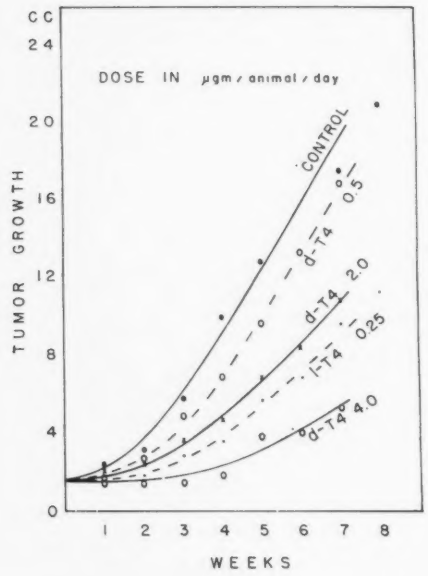


FIG. 2. The response of a transplantable pituitary tumor to 3 different doses of D-thyroxine compared with untreated control animals and those treated with 0.25 µg. of L-thyroxine.

compounds, ultimately attained large sizes. Doubling the dose of compound frequently flattened or depressed the growth curve for a few weeks. Growth then resumed at a rate similar to that observed before changing the dose. High initial doses of effective compounds were capable of completely suppressing tumor growth for the duration of the study.

Analogue effectiveness was calculated from the growth curve of the tumor seven weeks after injections started. This time was selected because beyond this point control animals often died. The average size of the tumors at 7 weeks was expressed as the percentage difference from the untreated control group. These were compared for a standard response to a group of animals treated with 0.25 μg. of L-thyroxine. Data obtained at 7 weeks from the effects of various concentrations of test analogues were plotted on semilog paper and the amount of compound necessary to induce a 50% depression in tumor growth determined. The calculated values, shown in Table 1, indicate the biological effectiveness of the compounds relative to L-thyroxine which was given an arbitrary value of 100. The calculated potency is based on the amount of compound, by weight, required to produce a 50% depression in tumor growth. The amount of L-thyroxine necessary to produce this degree of inhibition was found by repeated assay to range between 0.22 and 0.26 μg. per animal per day. No essential differences were found in the response of 5 different transplants of the same strain of tumor. Compounds were considered inactive if they failed to show inhibition of tumor growth at a maximal dose of 100 μg. per animal per day. This dose is about 400 times the dose of L-thyroxine required to produce a 50% inhibition of tumor growth.

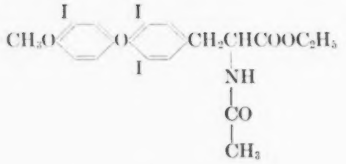
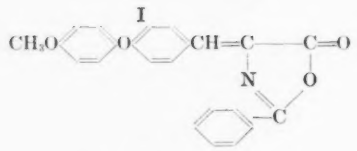
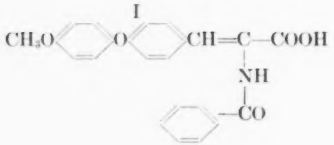
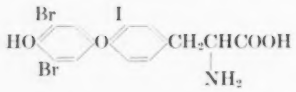
Of the 14 thyronine compounds studied only DL-thyroxine, D-thyroxine, 3,5,3′-triiodo-L-thyronine and n-acetyl-3,5-diiodo-4-(4′-methoxy-3′-iodophenoxy) phenyl alanine ethyl ester (W 1700) were found to have appreciable biological potency when compared to L-thyroxine (Table 1). Determination of potency, on a weight basis, showed DL-thyroxine to possess approximately one-half the effectiveness of L-thyroxine. L-Triiodothyronine (3,5,3′) on the other hand was found as effective as L-thyroxine. D-Thyroxine had 12% of the biological potency of L-thyroxine, or about one-fifth the activity of the racemic form. None of the moniodinated, diiodinated or other thyronines showed any inhibitory activity against tumor growth.

The two butyric acid analogues which were tested showed essentially no biological effect in preventing growth of the pituitary tumors.

Three propionic acid analogues were assayed and only the tetraiodinated and triiodinated compounds had significant activity against tumor growth. None of the 3 compounds, however, appeared to have more than 10% of the potency possessed by L-thyroxine.

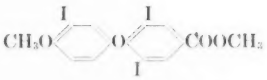
Four analogues of the thyroacetic acid series were studied and of these 3,5,3′,5′-tetraiodothyroacetic acid (tetrac) and 3,5,3′-triiodothyroacetic acid (triac) were found to be one-half as active as L-thyroxine. Reverse triac (3,3′,5′) showed no evidence of inhibition. The 3,5,3′-triiodothyro-

TABLE 1. RELATIVE POTENCY OF COMPOUNDS ON GROWTH OF TUMOR TRANSPLANTS

Compound		Relative potency
	Thyronine series	
	L-thyroxine	100
	D-thyroxine	12
	DL-thyroxine	56
	3,5,3′-triiodo-L-thyronine	100
	3,3′,5′-triiodo-D,L-thyronine	Inactive
	3,5,3′,5′-tetrachloro-D,L-thyronine	Inactive
W 1700*	 N-acetyl-3,5-diiodo-4-(4′methoxy-3′-iodophenoxy) phenyl alanine ethyl ester	25
No. 33	 4-[3′-iodo-4′-(4″-methoxy phenoxy)-benzal]-2-phenyl-5-oxazolone	Inactive
No. 34	 α-benzamido-3-iodo-₃4(4′-methoxy phenoxy)-cinnamic acid	Inactive
W 1631	3-iodo-D,L-thyronine	Inactive
W 1651	 3′,5′-dibromo-3-iodo-D,L-thyronine	Inactive
W 1641	3,3′-diiodo-D,L-thyronine	Inactive
No. 37	3,5-diiodo-L-thyronine	1
No. 12	1-nitro-5-(3,5-dinitro-thyromethyl-ene) hydantoin, methyl ether	Inactive
	Butyric acid series	
W 1620	3,5,3′,5′-tetraiodothyrobutyric acid	3
W 1621	3,5,3′-triiodothyrobutyric acid	Inactive
	Propionic acid series	
W 1524	3,5,3′,5′-tetraiodothyropropionic acid	5
W 1525	3,5,3′-triiodothyropropionic acid	10
W 1628	3,3′,5′-triiodothyropropionic acid	Inactive

* The structural formulas of compounds indicated by numbers only may be found in the paper of Stasilli, Kroc and Meltzer (7).

TABLE 1—*continued*

Compound			Relative potency
		Acetic acid series	
W 1483		3,5,3′,5′-tetraiodothyraocetic acid	50
W 1499		3,5,3′-triiodothyroacetic acid	50
W 1699		3,3′,5′-triiodothyroacetic acid	Inactive
W 1702		3,5,3′-triiodothyroacetic acid, methyl ether	7
		Formic acid series	
W 1489		3,5,3′,5′-tetraiodothyroformic acid	Inactive
W 1606		3,5-diiodo-4-(4′-hydroxyphenoxy)-benzoic acid	Inactive
No. 6		3,5-diiodothyroformic acid, methyl ether, methyl ester	Inactive
No. 11		3,5,3′-triaminothyroformic acid, methyl ether, methyl ester	Inactive
No. 14		methyl 3,5-diiodo-4-(3′iodo-4′-methoxy phenoxy)-benzoate	Inactive

acetic acid methyl ether (W 1702) had 10% the potency of L-thyroxine.

Five thyroformic compounds were investigated and all appeared to be devoid of biological effectiveness against the transplantable tumors.

DISCUSSION

This study confirms the observations of Furth and Gadsden (5), that thyroxine administration inhibited the growth of transplantable TSH secreting pituitary tumors in mice. From the present data it is clear that this inhibitory capacity is not limited to thyroxine since some thyroxine analogues were also capable of suppressing pituitary tumor growth. When the present data are compared with previously reported studies using essentially the same analogues it is apparent that no good correlation exists between prevention of tumor growth and tadpole metamorphosis (6), I^{131} collection by the rat thyroid (8) or oxygen consumption (7). The best correlation appears to exist between pituitary tumor depression and prevention of a thiouracil induced goiter (7).

If the relative potency for goiter prevention (7) is plotted against the inhibition of pituitary tumor growth for the effective compounds shown in Table 1, the majority of the points fit closely to the theoretical curve.

The two main exceptions are compound W 1700 and 3,5,3′-triiodo-L-thyronine, both of which are more effective in preventing a thiouracil induced goiter in rats than suppressing pituitary tumor growth in mice. Whether this represents a true separation of response or is only a reflection of variations which exist in the precision of the determinations (6, 7) is at present unknown. It may be of significance, however, that 3,5,3′-triiodo-L-thyronine was the only analogue found in the present study to be as potent as L-thyroxine. This is of interest since triiodothyronine has been found by the more classical methods of study to be 3 to 8 times more active than L-thyroxine. No compounds were found more effective in the present study than either thyroxine or triiodothyronine.

An important consideration regarding this type of study is that of reproducibility of results depending on the strain of pituitary tumor. It has been reported previously (3) that "dependent tumors" of the type used above are capable of becoming "autonomous" in the course of serial transplantation. Moreover, the TSH concentration of these tumors is also altered by successive transplantations (9). It was observed in the present study that two strains of tumors (LA3052A and B) showed marked quantitative differences in growth characteristics. For this reason, all comparative studies were done using the A strain of tumor. This tumor, in the two generations through which it has been carried, has given consistent and reproducible results. It has yet to be determined whether the response of other strains of tumors to analogues will differ.

The mechanism of the inhibition of tumor growth is not known. Since there is no direct anatomical connection with the hypothalamus, it can be assumed that the action is directly on the tumor cells. However, an indirect effect via some other pathway has not been ruled out.

Acknowledgments

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REFERENCES

1. Gorbman, A.: *Proc. Soc. Exper. Biol. & Med.* **71**: 237. 1949.
2. Furth, J. and W. T. Burnett, Jr.: *Proc. Soc. Exper. Biol. & Med.* **78**: 222. 1951.
3. Furth, J., E. L. Gadsden and W. T. Burnett, Jr.: *Proc. Soc. Exper. Biol. & Med.* **80**: 4. 1952.
4. Furth, J., J. N. Dent, W. T. Burnett, Jr. and E. L. Gadsden: *J. Clin. Endocrinol. & Metab.* **15**: 81. 1955.
5. Furth, J. and E. L. Gadsden: *Proc. Soc. Exper. Biol. & Med.* **83**: 511. 1953.
6. Money, W. L., R. I. Meltzer, J. Young and R. W. Rawson: *Endocrinology* **63**: 20. 1958.
7. Stasilli, N. R., R. L. Kroc and R. I. Meltzer: *Endocrinology* **64**: 62. 1959.
8. Money, W. L., R. I. Meltzer, D. Feldman and R. W. Rawson: *Endocrinology* **64**: 123. 1959.
9. Bates, R. W., E. Anderson and J. Furth: *Endocrinology* **61**: 549. 1957.

THE EFFECTS OF ESTROGEN ON UTERINE PHENOL-STIMULATED OXIDASE[1]

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ABSTRACT

Uterine phenol-stimulated DPNH oxidase is not present in spayed, spayed-adrenalectomized, hypophysectomized, or immature rats. Small doses of estrogens for three days greatly increase the activity of the enzyme system in all of these animals. The intracardiac administration of 10 μg. of estradiol produced a significant increase in enzymatic activity in 2 hours in spayed rats, 12 hours in immature and hypophysectomized rats, but failed to elicit activity in 12 hours in spayed-adrenalectomized animals. Rats weighing 110–160 gm. showed a cyclical correlation between enzyme activity and the vaginal smear but 185–240gm. rats did not show this correlation. The early increase in uterine weight due to water imbibition following the administration of estrogen is not related to enzymatic activity. Testosterone, cortisone, and progesterone did not enhance enzymatic activity in the rat uterus.

PREVIOUS studies from this laboratory have shown (1) that the uteri of intact and estrogen treated oophorectomized rats contain a reduced diphosphopyridine nucleotide (DPNH) oxidase[2] which requires as co-factor certain phenolic substances. The phenol-activated oxidase is markedly decreased in uterine homogenates from oophorectomized rats. This report presents further studies on the biological behavior of this enzyme.

MATERIALS AND METHODS

Sprague-Dawley female rats of various weights which will be indicated subsequently, as well as 6 week old hypophysectomized rats were obtained from The Charles River Breeding Laboratories. They were maintained on Purina Dog Checkers and water ad libitum. In addition hypophysectomized and adrenalectomized rats received a 5% dextrose and 1% sodium chloride drinking solution. The animal room was maintained at 24° C.

The enzymatic activities in this report are expressed as the change in optical density at 340 mμ per minute per mg. wet weight of tissue. This unit was felt to be preferable to expression of activity in terms of nitrogen for several reasons. Uterine tissue contains a high and variable amount of collagen depending on the functional state of the tissue (2). The uterus of the oophorectomized rat has an 80% water content which rises to a maximum of 85% six hours after the administration of estradiol and returns to 80%

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[2] Hereafter the terms oxidase or enzyme will refer to uterine phenol-activated oxidase.

water content in 72 hours (3). Almost the entire dry weight can be accounted for in terms of protein, so that it would not be anticipated that significant differences in enzyme activity would occur as a result of a generalized protein increase. Finally, evidence has been presented in a previous report that homogenates prepared on a comparable wet weight basis from the uteri of oophorectomized and estrogen treated rats do not exhibit a large difference in protein content (1).

Assay of phenol-activated oxidase

After the rats were sacrificed by a sharp blow on the head, a 5% homogenate of each rat uterus was prepared immediately in ice cold 0.25 M sucrose with the aid of a conical glass homogenizer supplied by the Kontes Glass Company. The homogenate was filtered through nylon gauze prior to use. The assay was performed spectrophotometrically by measuring the rate of disappearance of DPNH at 340 mμ in a Beckman model B spectrophotometer. The incubation mixture consisted of 0.006 M phosphate buffer pH 6.8, 5×10^{-5} M manganese chloride, 1×10^{-4} M DPNH, and appropriately diluted homogenate in a total volume of 3.0 cc. The initial rate was determined at room temperature in the presence and absence of 3×10^{-4} M 2,4-dichlorophenol. No oxidation of DPNH occurred in the absence of dichlorophenol. Appropriate volumes of homogenates were used for the assays so that the observed rates were proportional to the amount of enzyme added.

RESULTS

Effect of administration of estrogen

Figure 1 shows the effect of the administration of 3 daily doses of estradiol-17β subcutaneously in sesame oil to rats weighing 150 gm. These animals had been spayed one month previously. They were killed on the 4th day and the uteri were assayed. The level of enzyme in uterine homogenates from spayed animals increased from control values when the dose of estradiol was 0.5 μg. Above this dose little additional activity was observed. Comparable homogenates of liver, kidney, spleen, and adrenal showed no detectable oxidase activity.

A study similar to that performed on spayed rats was carried out on 150 gm. spayed-adrenalectomized animals as shown in Table 1. Surgery was done one month before the animals were given daily injections of estradiol for three days. Untreated spayed-adrenalectomized rats had no uterine oxidase activity. However, the administration of as little as 0.01 μg. of estradiol to these rats induced significant enzymatic oxidation of DPNH. Doubling the dose of estradiol increased further the enzymatic activity.

Table 2 illustrates the effect of three daily doses of estradiol to 25 day old rats. Untreated animals had no detectable oxidase except for one animal out of twenty which showed the barely significant level of 0.005 O.D./min./mg. tissue. Activity was not induced with 0.01 μg. of estradiol in these animals, but 0.025 μg. of estradiol induced significant oxidase activity which increased with increase in the dose. A duplicate experiment with the additional administration of 1.0 mg. of progesterone in oil on the second day produced enzymatic activities no higher than those achieved with estradiol alone. Six week old hypophysectomized rats had no de-

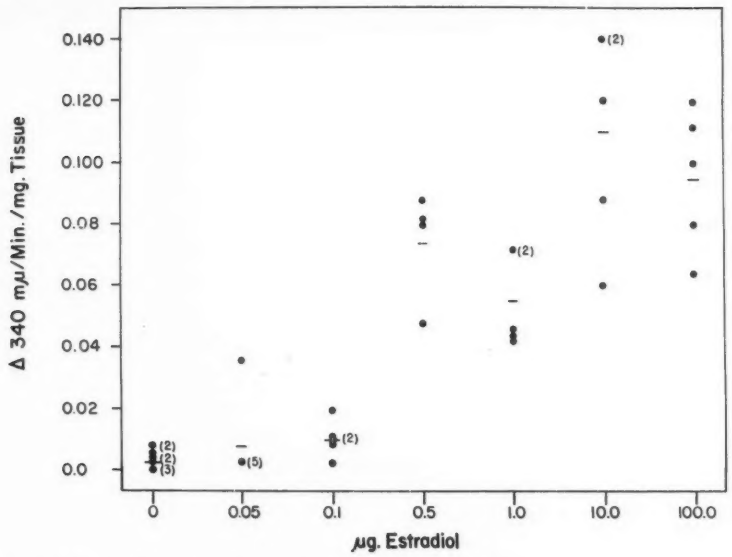

FIG. 1. Effect of various doses of estradiol in spayed rats on uterine oxidase. The hormone was given daily for 3 days by subcutaneous injection and the animals sacrificed on the fourth day. Numbers enclosed by ( ) indicate the number of rats showing an identical assay. At a dose of 0.5 μg. and higher the mean of the enzymatic activity differs significantly from the mean control value by a "t" test.

tectable levels of uterine oxidase and the administration of estradiol produced enzymatic levels somewhat less than that engendered in immature animals.

The time course of the induction of oxidase activity was studied by injecting intact, spayed, spayed-adrenalectomized and immature rats with 10 μg. of estradiol in 0.1 cc. propylene glycol by the intracardiac route. Figure 2 shows such a study in 150 gm. rats spayed one month before injection. In this experiment, a third of the control animals showed measurable enzymatic activity. At 2 and at 4 hours after injection, the enzymatic

TABLE 1. THE EFFECT OF ESTRADIOL ON UTERINE OXIDASE IN SPAYED-ADRENALECTOMIZED RATS

The indicated doses of estradiol were injected subcutaneously daily for 3 days and the rats sacrificed on the 4th day for assay.

Dose, μg.	Number of rats	O.D./min./mg. tissue, mean ±S.E.M.
0.000	6	0.000
0.010	5	0.007 ±0.002
0.025	4	0.020 ±0.005
0.200	5	0.039 ±0.006
1.000	5	0.027 ±0.007

TABLE 2. THE EFFECT OF ESTRADIOL ON UTERINE OXIDASE IN 25 DAY OLD RATS

The indicated doses of estradiol-17β were injected subcutaneously daily for 3 days and the rats sacrificed on the 4th day for assay.

Dose, μg.	Number of rats	O.D./min./mg. tissue, mean $\pm$S.E.M.
0.000	20	0.00
0.005	4	0.00
0.010	4	0.00
0.025	4	0.034 $\pm$0.015
0.100	4	0.048 $\pm$0.010
0.200	4	0.069 $\pm$0.005
100.0	4	0.120 $\pm$0.005

activity was increased over the controls by a barely significant ($P < 0.05$) amount. Within 72 hours oxidase activity had declined to control levels. Several of the control spayed rats showed significant oxidase activity without, however, concomitant uterine enlargement. It was further noted that those uteri which remained inactive with respect to enzyme function at 2 and 4 hours after estrogen injection (cf. Fig. 2) nevertheless grossly appeared to have been stimulated by the hormone. Table 3 shows the result of intracardiac administration of estradiol to 25 day old rats. Four hours after injection, the uterus had responded with the expected weight in-

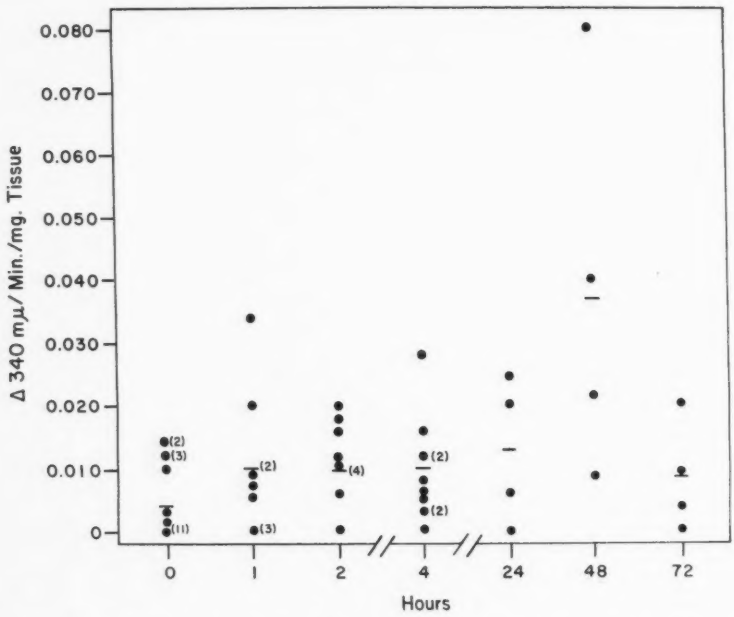

FIG. 2. Effect of intravenous estradiol on uterine oxidase in spayed rats. The animals were sacrificed at the indicated times after intracardiac injection of 10 μg. of estradiol in 0.1 cc. propylene glycol.

TABLE 3. THE EFFECT OF INTRACARDIAC ADMINISTRATION OF ESTRADIOL ON UTERINE OXIDASE IN IMMATURE RATS

Ten μg. of estradiol-17β were injected by the intracardiac route the indicated number of hours before the enzyme assay.

Time in hours	Number of rats	Uterine weight in mg., mean ±S.E.M.	O.D./min./mg. tissue, mean ±S.E.M.
0	9	40 ± 7	0.00
2	4	49 ± 5	0.00
4	4	66 ± 7	0.00
8	4	60 ± 2	0.006 ±0.003
12	6	58 ± 7	0.009 ±0.003
24	4	84 ± 5	0.010 ±0.007
48	4	132 ±13	0.021 ±0.002
80	3	157 ±12	0.038 ±0.003

crease, but the enlarged hyperemic uteri were devoid of enzyme activity. At 8 and 12 hours, half of the animals showed significant activity, and at 24, 48, and 80 hours all manifested definite enzyme activity. These experiments indicate that the uterus may increase in weight as a result of estrogen stimulation without a necessary gain in oxidase activity.

Preliminary studies showed that the intracardiac administration of 10 μg. of estradiol to spayed-adrenalectomized rats did not give rise to perceptible levels of enzyme in 12 hours. For this reason, the effect of this dose of estrogen was studied in spayed-adrenalectomized rats which had been previously treated with small "priming" doses of estrogen in the hope that the enzyme system would be induced more rapidly. Rats weighing 150 gm. were spayed and adrenalectomized one month prior to the experiment. Three days prior to the experiment the daily injection of 0.01 μg. of estradiol in oil was begun. On the fourth day, an intracardiac injection of 10 μg. of estradiol was made. Table 4 demonstrates that the early induction of enzymatic activity did not occur in these animals. In this study, the increased uterine weight which occurred rapidly in response to the intracardiac dose is in striking contrast to the negligible enzymatic levels obtained.

Experiments were done to determine the effect of intracardiac adminis-

TABLE 4. THE EFFECT OF INTRACARDIAC ADMINISTRATION OF ESTRADIOL ON UTERINE OXIDASE IN SPAYED-ADRENALECTOMIZED RATS

The data marked by 0* represents untreated spayed-adrenalectomized animals. The remainder of the data was obtained from spayed-adrenalectomized rats treated with 0.01 μg. of estradiol administered for 3 days as a subcutaneous injection in oil. On the 4th day intracardiac injection was done of 10 μg. of estradiol in propylene glycol. The time column represents hours after intracardiac injection.

Time in hours	Number of rats	Uterine weight in mg., mean ±S.E.M.	O.D./min./mg. tissue, mean ±S.E.M.
0*	7	181 ± 9	0
0	5	181 ±15	0.007 ±0.002
2	4	254 ± 5	0.005 ±0.002
4	4	269 ±33	0.002 ±0.002
8	4	262 ±17	0.007 ±0.001

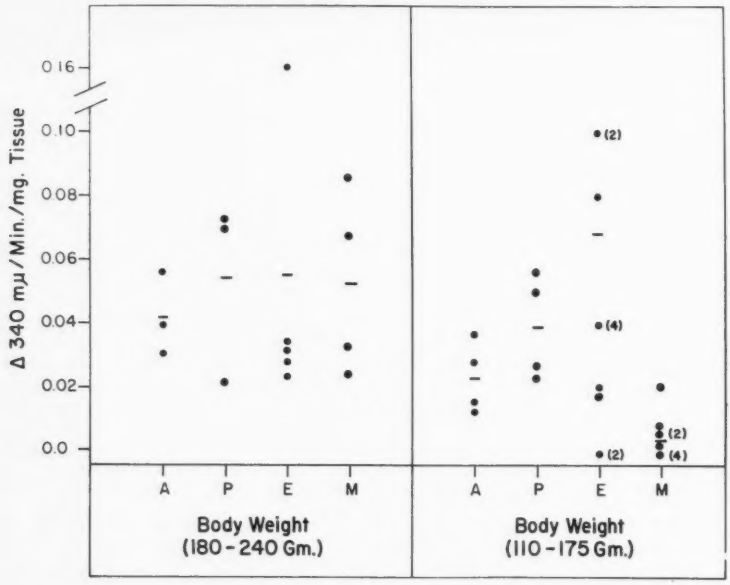

FIG. 3. Uterine oxidase levels in intact rats. The animals were divided into two groups on the basis of body weight as shown. Smears were coded as follows: A, anestrous; P, proestrous; E, estrous; M, metestrous.

TABLE 5. THE EFFECT OF VARIOUS COMPOUNDS ON UTERINE ENZYME ACTIVITY

All compounds were given daily in the indicated doses by subcutaneous injection for three days. The animals were sacrificed on the 4th day for uterine enzyme assay.

Compound	Dose, μg.	Number of rat	Type of rat	Uterine weight in mg., mean ±S.E.M.	O.D./min./mg. tissue, mean ±S.E.M.
Control	0	10	immature	39 ± 7	0.000 ±0.00
Control	0	17	spayed	111 ± 5	0.003 ±0.002
Estradiol-17β	0.10	5	spayed	246 ±15	0.011 ±0.005
Estriol	0.10	5	spayed	161 ±20	0.007 ±0.003
Estriol*	100	2	spayed	308 ± 0	0.056 ±0.010
Progesterone	1000	4	spayed	152 ±15	0.009 ±0.002
Progesterone	100	4	immature	47 ± 7	0.000 ±0.00
Stilbestrol	0.005	4	immature	119 ±10	0.000 ±0.00
Stilbestrol	0.010	4	immature	165 ±25	0.005 ±0.002
Stilbestrol	0.025	2	immature	202 ± 3	0.022 ±0.002
Norethynodrel	10.0	2	immature	69 ± 3	0.000 ±0.00
17-Ethinyl-19-nortestosterone	1000	3	immature	98 ± 5	0.000 ±0.00
Estradiol-17β + 17-Ethinyl-19- nortestosterone	3.0 100	2	immature	145 ± 3	0.014 ±0.002
Testosterone	1000	6	spayed	247 ±12	0.000 ±0.00
Cortisone	1000	2	spayed	100 ± 5	0.000 ±0.00

* A single injection was given 72 hours before assay.

tration of estradiol on hypophysectomized rats. Enzyme activity was present 12 hours after intracardiac administration of 10 μg. of estradiol to hypophysectomized rats and absent in untreated controls.

Uterine enzyme levels in intact rats

In contrast to uterine homogenates from spayed or hypophysectomized rats, preparations from intact females showed definite enzymatic activity. Vaginal smears were done on 40 rats selected at random from the animal room, and the corresponding uterine homogenate assayed for enzyme activity. Figure 3 shows the data when rats were separated into two groups, 100–175 gm., and 180–240 gm. in body weight. When the vaginal smear pictures were broken down into anestrus, proestrus, estrus, and metestrus, it became apparent that the uteri of the younger rats showed minimal activity during metestrus. In this group of animals the uterine activity varied in a cyclical fashion reaching a maximum during estrus. In the older group of rats examined there was a moderate level of uterine oxidase activity regardless of the vaginal smear picture and no cyclical variation was seen.

Effect of various compounds on the induction of the oxidase

The effect of various hormones on the induction of oxidase activity is shown in Table 5. Estradiol and stilbestrol induced definite enzymatic activity. Progesterone gave an insignificant increase at a high dose level. Testosterone, cortisone, norethynodrel,[3] and 17α-ethinyl nortestosterone produced no effect on the oxidase activity, although all except cortisone increased the uterine weight.

DISCUSSION

Phenol-activated oxidation of reduced diphosphopyridine nucleotide cannot be detected by the present technique in the uterus of hypophysectomized, spayed-adrenalectomized, or immature rats. The explanation for the occurrence of the system in uteri from occasional spayed rats is not clear. Most spayed rats have no detectable level of the enzyme and the probable explanation for the exceptions lies in a varying degree of adrenal estrogen production. Woolley has shown that estrogen is produced by the adrenals of certain strains of spayed mice (4). This question is still under study. The dose of estrogen required to produce significant levels of uterine oxidase is in the range of that used to elicit physiological response. There is no satisfactory explanation for the lack of response of an occasional animal to doses of estradiol capable of producing marked effects in most animals. The induction of oxidase over a three day period of injection with estrogen in the spayed-adrenalectomized animal does not require adrenal or hypo-

[3] Norethynodrel-17α-ethinyl-17 hydroxy-5(10)-estraen-3-one.

physeal function and occurs promptly in immature animals just as it does in mature spayed females.

The rapid development of oxidase activity following the intracardiac injection of a single dose of estradiol to spayed rats is of interest. Within two hours, a significant increase in enzyme activity has occurred. This increase occurred during the period of concomitant rapid water imbibition (5) and enhanced oxygen consumption (6) due to the administration of estradiol to the castrate rat. During this early period, serine aldolase activity increases slightly (7).

The rapid induction of oxidase activity in spayed animals resulting from the intracardiac administration of estradiol suggested that the enzyme might play a key role in the early phase of estrogen induced uterine growth. However, the failure of early enzyme induction by the administration of estrogen in spayed-adrenalectomized and immature animals demonstrates that this hypothesis is untenable since early uterine weight increase occurred in these studies. Evidence has been presented by Spaziani and Szego that the early response of the uterus to estrogen can be explained by a histamine mechanism (8, 9).

The cylical variation in uterine oxidase in young mature rats suggests a possible physiological role for this enzyme system. Studies by Ring have shown that alkaline phosphatase also undergoes a cyclical variation in activity in the uterus dependent on the phase of estrus (10). No explanation is afforded for the lack of cyclical enzymatic activity in the uterus of the heavier rats. These animals were not senescent, but varied in age from 7 to 12 weeks, nor had they ever been pregnant. Perhaps enough adrenal estrogen is produced at a certain stage of development to prevent a cyclical decline in oxidase. Further study is required to elucidate this question.

Stilbestrol and estradiol differ appreciably in chemical structure but are both estrogenic and both are capable of oxidase induction. The administration of testosterone produced considerable increase in uterine weight but gave rise to no oxidase activity. Norethynodrel and 17α-ethinyl-19-nortestosterone also induced uterine growth without oxidase induction. Edgren demonstrated that norethynodrel had a steep slope in the dose response curve of uterine weight (11). It thus behaved like an estrogen in contrast to 17α-ethinyl-nortestosterone which showed a shallow response curve typical of impeded estrogen (12, 13). It is therefore somewhat suprising that norethynodrel failed to induce enzyme activity. In general, however, it appears that estrogenic substances increase uterine oxidase activity while other steroids, even though they are capable of increasing uterine weight, fail to affect this enzyme.

Uterine phenol-stimulated oxidase bears certain resemblances to the uterine DPNH oxidase system described by Bever *et al.* (14). The daily administration of estrogen for 3 days increased the activity of this system about fourfold. Untreated castrated animals had low but definite DPNH

oxidase activity. However, this enzyme was measured at pH 8.2 in the presence of cyanide. Phenol-stimulated oxidase has almost no activity at this pH and is completely inhibited at the concentration of cyanide used. No phenolic or other cofactor requirement was evident in the system studied by these authors.

Uterine phenol-stimulated oxidase has features in common with the uterine peroxidase of the rat described by Lucas *et al.* (15). The latter enzyme catalyzes the oxidation of certain reduced dyes by hydrogen peroxide. It is almost absent in the uterus of spayed animals and shows a dramatic increase upon the administration of estrogen. Akazawa and Conn (16) have shown that horseradish peroxidase has DPNH oxidase activity in the presence of certain phenols.

If the oxidase has physiological significance, the phenolic cofactor in tissues remains to be identified. Efforts to isolate such a factor have thus far been unsuccessful. Estradiol has been shown to serve as a phenolic cofactor with lacto-peroxidase, horseradish peroxidase and phenolase (17). Estradiol can be shown to serve as cofactor for the oxidase under somewhat different conditions from those involving dichlorophenol (18). However, estradiol catalyzes the reaction less efficiently than does dichlorophenol and may not represent the cofactor of uterine tissue.

REFERENCES

1. Hollander, V. P. and M. L. Stephens: *J. Biol. Chem.* **234:** 1901. 1959.
2. Needham, D. M. and J. M. Cawkwell: *Biochem. J.* **65:** 540. 1957.
3. Telfer, M.: *Arch. Biochem. Biophys.* **44:** 111. 1953.
4. Woolley, G., E. Fekete and C. C. Little: *Proc. Natl. Acad. Sci. U. S.* **25:** 277. 1939.
5. Astwood, E. B.: *Endocrinology* **23:** 25. 1938.
6. Roberts, S. and C. M. Szego: *J. Biol. Chem.* **201:** 21. 1953.
7. Herranen, A. and G. C. Mueller: *Biochem. and Biophys. Acta* **24:** 223. 1957.
8. Spaziani, E. and C. M. Szego: *Endocrinology* **63:** 669. 1958.
9. Spaziani, E. and C. M. Szego: *Endocrinology* **64:** 713. 1959.
10. Ring, J. R.: *Anat. Rec.* **107:** 121. 1950.
11. Edgren, R. A.: *Endocrinology* **62:** 689. 1958.
12. Huggins, C. and E. V. Jensen: *J. Exp. Med.* **102:** 335. 1955.
13. Pincus, G., M. C. Chang, M. Zarrow, E. Hafez and A. Merrill: *Endocrinology* **59:** 695. 1956.
14. Bever, A. T., J. T. Velardo and F. L. Hisaw: *Endocrinology* **58:** 512. 1956.
15. Lucas, F. V., H. A. Neufeld, J. G. Utterback, A. P. Martin and E. Stotz: *J. Biol. Chem.* **214:** 775. 1955.
16. Akazawa, T. and E. E. Conn: *J. Biol. Chem.* **232:** 403. 1958.
17. Williams-Ashman, H. G., M. Cassman and M. Klavins: *Fed. Proc.* **18:** 352. 1959.
18. Temple, S., V. P. Hollander, N. Hollander and M. L. Stephens (In Preparation).

CORTISOL-4-C^{14} IN THE ADRENAL OF THE NORMAL AND VITAMIN C DEFICIENT GUINEA PIG[1]

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ABSTRACT

Metabolism of cortisol-4-C^{14} has been studied in normal and in vitamin C deficient guinea pigs. Cortisol-4-C^{14} and its metabolites were found in the adrenal gland after the intravenous injection of labeled cortisol. Compared to normal animals, guinea pigs that have been on ascorbic acid deficient diet for 7 or 14 days showed a more rapid loss of the labeled material from the adrenal gland. This rapid disappearance was unique for the adrenal gland and was not observed in plasma, liver, kidney, spleen, and thymus of vitamin C deficient animals. This finding supports the idea that vitamin C may effect synthesis, exchange, and release of cortisol in the adrenal cortex.

STEROIDOGENESIS by the adrenal cortex has been associated with a decrease in the high ascorbic acid concentration of this tissue (1, 2). Thus Sayers' assay for ACTH was based upon the decrease of adrenal ascorbic acid in the hypophysectomized rat. In the guinea pig the depression of ascorbic acid following ACTH administration was more prolonged than in the rat (3), perhaps due to the inability of the guinea pig to synthesize ascorbic acid. Some doubt has been placed upon the role of ascorbic acid in the synthesis of adrenal corticosteroids by the finding of high levels of plasma 17-hydroxycorticosticosteroids (17-OHCS) in guinea pigs with advanced scurvy (4). Both the decrease in adrenal ascorbic acid during the augmented corticosteroidogenesis in the guinea pig and the elevated plasma 17-OHCS in the ascorbic acid depleted animal may be explained by the hypothesis that ascorbic acid may act as a "brake" in the synthesis or release of corticosteroids (5).

In previous studies (6) the adrenocortical function of guinea pigs maintained on a vitamin C deficient diet has been investigated by a) determining 17-OHCS in plasma and bile following the injection of ACTH and bacterial polysaccharide, b) measuring the clearance of cortisol-4-C^{14} from blood plasma. Prior to the onset of loss of body weight, the ascorbic acid concentration in the adrenal gland of these animals was markedly decreased, but no significant change was observed in the clearance of cortisol-

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4-C^{14} from the plasma. Such animals responded normally to both ACTH and bacterial polysaccharide.

The present study extends these investigations to distribution of C^{14} in different tissues of normal and vitamin C deficient animals following the intravenous administration of cortisol-4-C^{14}.

MATERIAL AND METHODS

Seventy-two young growing guinea pigs of either sex, weighing 150–250 gm. were divided into three groups with 24 animals per group. The control group was maintained on a diet of Purina rabbit chow with daily supplements of cabbage. The other two groups were fed the ascorbic acid deficient diet of Rinehart and Mettier (7) for 7 and 14 days, respectively. The animals were weighed every second day and were still growing at the 12th day of the diet. None of the vitamin C deficient animals were seriously ill at the time of the investigation. Histologic examination of the costochondral and tibial epiphyses was used to verify vitamin C deficiency (6).

Normal guinea pigs and those maintained on vitamin C deficient diet for 7 days were given 0.045 μC = 0.0116 μg. cortisol-4-C^{14}/100 gm. of body weight intravenously in 0.1 ml. 0.85% sodium chloride. The animals fed the ascorbic acid deficient diet for 14 days received 0.180 μC = 0.0464 μg. cortisol-4-C^{14}/100 gm. of body weight in 0.1 ml. 0.85% sodium chloride. In each group 4 animals were sacrificed at $\frac{1}{2}$, 1, 2, 4, 6, and 9 hours after the injection of labeled cortisol.

The animals were killed with a neck clamp technique (8). Blood was withdrawn by cardiac puncture into a heparinized syringe, and plasma separated by centrifugation, diluted with an equal volume of 0.85% sodium chloride, and immediately extracted 3 times with methylene dichloride (v:v), followed by 3 extractions with n-butyl alcohol (v:v). Acid hydrolysis in 10% hydrochloric acid at 80° C was finally performed and the hydrolysates extracted with n-butyl alcohol (3 times, v:v). C^{14} was determined by a windowless flow counter in all extracts. Counting efficiency in proportional region was 50%. Sufficient counts were accumulated to afford a 1% standard error for most samples and 3% for samples of lowest C^{14} content.

Adrenal, kidney, liver, spleen, thymus were removed from all animals, frozen, and later processed the same way as outlined for the blood plasma. The tissues were, however, homogenized before being extracted. Each tissue was processed separately except for those from the 7 day vitamin C deficient group. In this group each tissue was pooled according to time.

RESULTS

Plasma

The total concentration of C^{14} in the plasma fell rapidly during the first thirty minutes after the injection of cortisol-4-C^{14}. Following this rapid initial fall, the total activity recovered from plasma showed approximately the same decrease in the vitamin C deficient animals as in the control group (Fig.1, Table 1). The methylene dichloride extractable labeled material showed a similar clearance in all three groups (Fig. 3, Table 1). The concentration of C^{14} in the first butanol fraction remained fairly constant throughout the experiment in all three groups (Fig. 4, Table 1). All radioactivity in the plasma was recovered in methylene dichloride and in first butanol fraction in normal guinea pigs and in animals on deficient diet for 7 days. However, some C^{14} was found in the acid hydrolyzable fraction in

animals which had been on a vitamin C deficient diet for 14 days (Fig. 5, Table 1).

Adrenal

The amount of C^{14} recovered from the adrenals was relatively high and

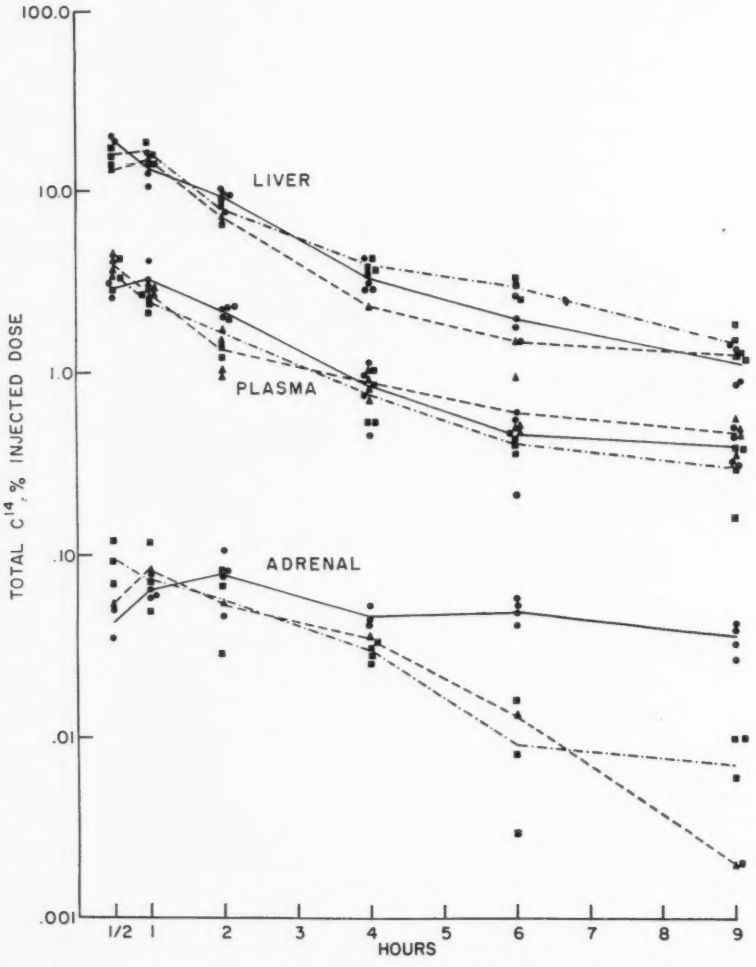

FIG. 1. Total C^{14} from liver, plasma, and adrenal following the intravenous administration of cortisol-4-C^{14} into normal animals and animals on vitamin C deficient diet for 7 and 14 days. The amounts of activity are expressed in % of injected cortisol-4-C^{14} and plotted semilogarithmically against time after injection.

●——● average curve from normal animals.
▲- - - - -▲ average curve from animals 7 days on vitamin C deficient diet.
■-·-·-·-·-■ average curve from animals 14 days on vitamin C deficient diet.

remained elevated despite the rapid disappearance from plasma, liver, kidney, and spleen (Fig. 1, Table 1) in normal animals. In contrast, the vitamin C deficient groups showed an adrenal clearance of C^{14} in excess of that of other tissues. This increased disappearance was manifested both in the methylene dichloride (Fig. 3, Table 1) and in the first butanol fraction (Fig. 4, Table 1). C^{14} extractable with butanol after acid hydrolysis did not vary

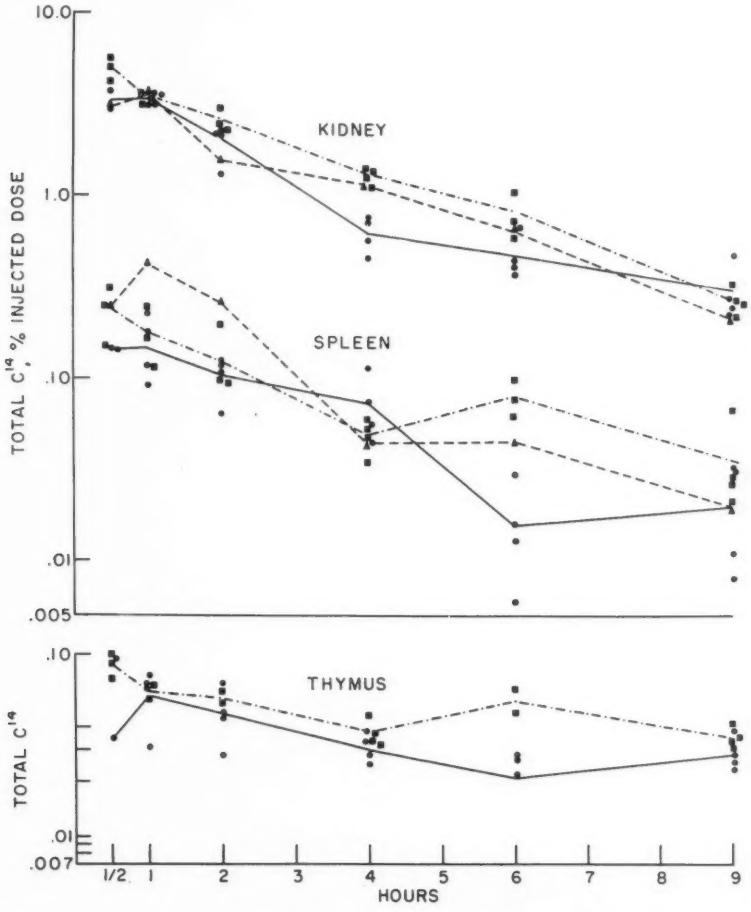

FIG. 2. Total C^{14} from kidney, spleen, and thymus following the intravenous administration of cortisol-4-C^{14} into normal animals and animals on vitamin C deficient diet for 7 and 14 days. The amounts of activity are expressed in % of injected cortisol-4-C^{14} and plotted semilogarithmically against time after injection.

●——● average curve from normal animals.
▲- - - - -▲ average curve from animals 7 days on vitamin C deficient diet.
■-·-·-·-·-■ average curve from animals 14 days on vitamin C deficient diet.

TABLE 1. MEAN C^{14} CONCENTRATIONS IN 100 ML. PLASMA OR 100 GM. TISSUE IN NORMAL AND VITAMIN C DEFICIENT GUINEA PIGS FOLLOWING THE INTRAVENOUS ADMINISTRATION OF CORTISOL-4-C^{14}. THE CONCENTRATIONS OF C^{14} ARE EXPRESSED IN % OF INJECTED CORTISOL-4-C^{14}

Time after injection	Fraction	Plasma			Adrenal			Kidney		
		Normal	7 days	14 days	Normal	7 days	14 days	Normal	7 days	14 days
½ hr.	Total	23.9 ± 1.3*	43.0 ± 1.7	35.2 ± 5.2	58.7 ± 3.6	68.4	96.3 ± 32.6	149.3 ± 22.2	94.5	203.7 ± 23.9
	CH_2Cl_2	24.0 ± 1.0	42.0 ± 2.6	30.7 ± 5.0	23.1 ± 0.2	32.1	73.4 ± 25.6	130.4 ± 19.7	70.1	155.1 ± 21.1
	Butanol	2.9 ± 0.3	1.0 ± 0.4	3.2 ± 0.6	6.2	31.4	21.8 ± 11.1	14.7 ± 1.9	19.4	37.6 ± 17.6
	Ac. hydr.	—	—	1.3 ± 0.1	32.5 ± 6.5	4.9	1.1 ± 0.4	8.3 ± 0.6	5.0	11.0 ± 3.0
1 hr.	Total	26.1 ± 1.5	27.6 ± 1.4	28.3 ± 1.8	74.7 ± 11.0	77.2	61.1 ± 16.4	141.2 ± 21.2	105.8	173.3 ± 33.6
	CH_2Cl_2	23.8 ± 2.0	25.3 ± 3.5	22.0 ± 10.0	11.2 ± 2.1	43.0	36.1 ± 14.6	111.0 ± 13.3	58.9	130.9 ± 29.5
	Butanol	2.3 ± 0.7	2.3 ± 0.8	4.5 ± 0.8	31.0 ± 8.8	30.3	18.7 ± 0.7	24.3 ± 6.3	38.3	34.3 ± 2.5
	Ac. hydr.	—	—	1.8 ± 0.9	32.4 ± 7.7	3.9	6.6 ± 3.0	8.4 ± 2.4	8.6	8.1 ± 2.2
2 hr.	Total	22.1 ± 5.2	13.3 ± 4.4	18.3 ± 4.9	105.5 ± 25.6	26.9	52.4 ± 13.3	102.5 ± 17.9	40.2	114.4 ± 15.1
	CH_2Cl_2	19.0 ± 4.3	11.2 ± 2.9	10.4 ± 2.5	28.5 ± 10.0	8.6	24.6 ± 7.0	70.9 ± 4.9	21.3	74.4 ± 9.6
	Butanol	3.1 ± 1.1	2.1 ± 0.9	6.3 ± 0.3	34.3 ± 15.2	13.4	15.1 ± 6.0	22.8 ± 7.9	12.4	30.6 ± 3.9
	Ac. hydr.	—	—	1.6 ± 0.4	42.6 ± 5.8	4.9	12.7 ± 4.6	8.2 ± 2.3	6.5	9.4 ± 1.5
4 hr.	Total	9.7 ± 2.8	7.3 ± 1.6	8.7 ± 2.9	77.4 ± 32.8	36.0	25.6 ± 1.3	39.1 ± 15.5	31.7	52.8 ± 6.7
	CH_2Cl_2	6.8 ± 2.5	5.2 ± 0.5	3.7 ± 2.3	26.2 ± 12.6	12.1	8.5 ± 1.4	20.8 ± 13.1	18.6	29.4 ± 4.6
	Butanol	2.8 ± 1.1	2.1 ± 1.2	3.7 ± 0.4	28.7 ± 12.5	17.2	9.7 ± 1.3	13.6 ± 2.8	10.1	18.7 ± 5.2
	Ac. hydr.	—	—	1.3 ± 0.3	22.5 ± 8.4	6.7	7.4 ± 1.1	4.5 ± 1.7	3.0	4.7 ± 1.0
6 hr.	Total	5.7 ± 1.9	5.3 ± 2.1	4.6 ± 1.0	64.9 ± 12.5	9.8	8.3 ± 4.4	25.4 ± 6.3	15.1	35.7 ± 1.6
	CH_2Cl_2	3.2 ± 1.6	2.9 ± 0.6	1.5 ± 0.3	24.9 ± 1.7	2.9	2.5 ± 1.0	12.5 ± 4.5	9.2	14.8 ± 2.2
	Butanol	2.5 ± 0.7	2.4 ± 1.5	2.5 ± 0.7	18.8 ± 6.5	2.8	4.8 ± 3.1	10.3 ± 3.1	5.1	18.1 ± 2.6
	Ac. hydr.	—	—	0.6 ± 0.2	21.2 ± 6.6	4.1	3.4 ± 1.2	2.6 ± 1.0	0.8	2.8 ± 0.4
9 hr.	Total	3.9 ± 1.0	3.5 ± 1.2	3.2 ± 1.4	44.3 ± 15.8	2.0	7.9 ± 1.4	14.0 ± 4.7	5.2	11.6 ± 1.8
	CH_2Cl_2	1.1 ± 0.7	1.1 ± 0.1	1.3 ± 0.9	18.2 ± 5.5	—	—	3.5 ± 0.2	1.5	4.2 ± 0.8
	Butanol	2.8 ± 1.2	2.4 ± 0.5	1.5 ± 0.3	6.3 ± 3.7	—	—	5.7 ± 2.7	2.5	5.3 ± 1.5
	Ac. hydr.	—	—	0.4 ± 0.2	19.8 ± 8.0	2.0	7.9 ± 1.4	4.8 ± 1.9	1.2	2.1 ± 1.0

* One standard deviation.

TABLE 1—*Continued*

Time after injection	Fraction	Liver			Spleen			Thymus	
		Normal	7 days	14 days	Normal	7 days	14 days	Normal	14 days
½ hr.	Total	166.3 ± 16.2	139.9	151.9 ± 15.2	33.4 ± 4.3	82.1	56.8 ± 14.9	11.4	38.8 ± 7.1
	CH_2Cl_2	119.9 ± 15.3	95.9	110.4 ± 10.0	31.4 ± 6.3	63.3	47.7 ± 11.2	6.1	23.3 ± 3.8
	Butanol	39.1 ± 1.4	33.9	33.2 ± 2.3	—	17.8	4.9 ± 3.4	5.3	7.6 ± 2.5
	Ac. hydr.	7.2 ± 0.5	10.1	8.3 ± 0.7	4.0	1.0	4.2 ± 0.4	—	7.9 ± 1.9
1 hr.	Total	121.4 ± 12.6	139.9	208.8 ± 44.2	44.6 ± 15.5	95.1	74.4 ± 36.8	20.1	39.3 ± 0.5
	CH_2Cl_2	77.2 ± 13.2	94.6	144.3 ± 34.8	23.2 ± 6.6	56.0	54.2 ± 32.6	13.7 ± 9.3	24.7 ± 2.5
	Butanol	34.9 ± 4.8	33.9	49.3 ± 4.5	10.9 ± 4.3	26.1	11.9 ± 3.9	3.9 ± 0.7	9.0 ± 2.0
	Ac. hydr.	9.2 ± 2.8	11.4	15.2 ± 2.1	10.4 ± 6.1	13.0	8.3 ± 0.8	—	5.5 ± 1.0
2 hr.	Total	95.1 ± 15.0	75.6	96.7 ± 16.0	38.3 ± 5.3	76.0	30.4 ± 6.5	13.1 ± 3.2	40.9 ± 12.0
	CH_2Cl_2	61.8 ± 9.7	43.9	63.0 ± 15.2	15.0 ± 3.2	15.5	25.3 ± 5.8	9.3 ± 3.1	20.7 ± 5.5
	Butanol	25.5 ± 3.5	20.7	25.8 ± 3.5	12.4 ± 2.6	5.9	2.5 ± 0.2	3.8 ± 0.4	6.0 ± 2.3
	Ac. hydr.	7.8 ± 2.8	11.0	7.9 ± 1.1	10.9 ± 3.5	54.6	2.6 ± 0.9	—	14.2 ± 4.2
4 hr.	Total	35.5 ± 7.1	29.5	44.4 ± 9.5	24.0 ± 5.1	13.9	15.2 ± 2.8	11.0 ± 3.0	22.1 ± 5.1
	CH_2Cl_2	13.3 ± 5.9	13.0	23.9 ± 8.0	10.0 ± 1.1	3.7	7.5 ± 3.8	7.5 ± 1.6	13.5 ± 2.8
	Butanol	11.6 ± 2.1	11.8	16.2 ± 2.5	7.5 ± 2.0	9.6	6.0 ± 3.9	3.5 ± 1.3	4.9 ± 2.0
	Ac. hydr.	6.3 ± 1.1	4.7	4.3 ± 1.0	6.4 ± 3.5	0.6	1.7 ± 1.4	—	3.7 ± 1.5
6 hr.	Total	24.5 ± 3.9	13.8	34.3 ± 10.4	5.3 ± 3.9	12.0	22.2 ± 8.5	7.0 ± 3.8	22.0 ± 4.5
	CH_2Cl_2	8.0 ± 2.1	2.7	16.0 ± 3.6	—	3.5	14.8 ± 5.4	1.8 ± 1.3	11.3 ± 3.1
	Butanol	11.8 ± 2.0	6.4	14.4 ± 6.9	5.3 ± 3.9	5.3	4.8 ± 3.8	5.2 ± 2.5	9.1 ± 0.7
	Ac. hydr.	4.7 ± 1.5	4.7	3.9 ± 0.4	—	3.2	2.6 ± 2.7	—	1.6 ± 0.6
9 hr.	Total	10.7 ± 2.4	11.5	15.7 ± 2.0	7.0 ± 3.7	1.5	9.5 ± 4.8	7.2 ± 0.6	18.2 ± 5.2
	CH_2Cl_2	2.6 ± 1.4	2.2	7.7 ± 3.8	0.7	—	2.8 ± 1.2	2.5 ± 0.9	9.1 ± 4.0
	Butanol	4.9 ± 1.3	5.5	4.4 ± 0.8	6.3 ± 3.7	1.5	5.0 ± 3.4	4.8 ± 1.1	6.6 ± 1.3
	Ac. hydr.	3.1 ± 0.8	3.8	3.6 ± 0.7	—	—	1.7 ± 1.9	—	2.5 ± 1.0

significantly throughout the experiment, although the amount found was lower in both vitamin C deficient groups than in control animals (Fig. 5, Table 1).

Liver, kidney, and spleen

The total C^{14} in liver and kidney was higher than in plasma, but the disappearance was similar. There was no significant difference between the three groups studied (Fig. 1 and 2, Table 1). The concentrations obtained by solvent fractionation of spleen homogenates were, however, more vari-

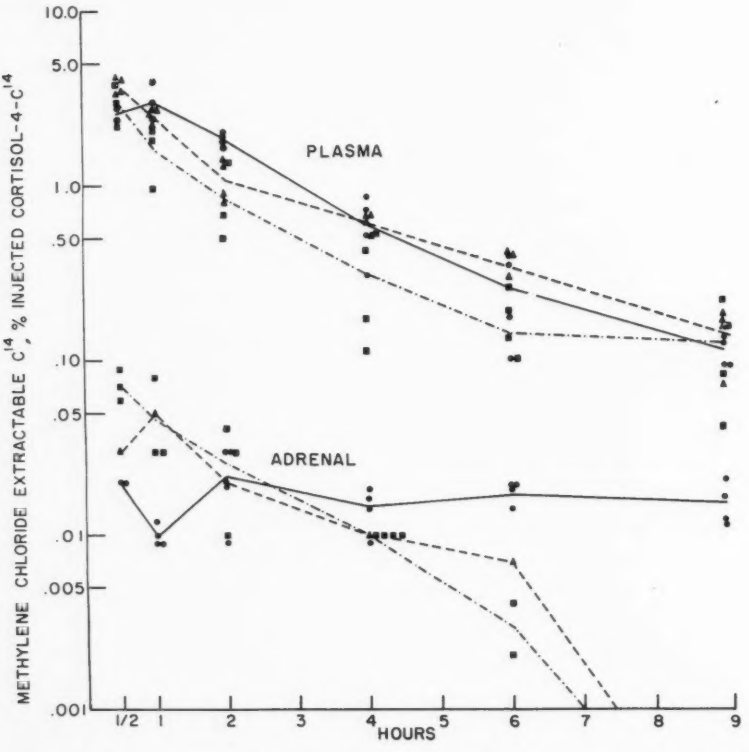

FIG. 3. Methylene dichloride extractable C^{14} from plasma* and adrenal following the intravenous administration of cortisol-4-C^{14} into normal animals and animals on vitamin C deficient diet for 7 and 14 days. The amounts of activity are expressed in % of injected cortisol-4-C^{14} and plotted semilogarithmically against time after injection.

●——● average curve from normal animals.
▲- - - -▲ average curve from animals 7 days on vitamin C deficient diet.
■-·-·-·-■ average curve from animals 14 days on vitamin C deficient diet.

* The plasma values of normal animals and animals 7 days on a vitamin C deficient diet have already been published (6).

able than those of the other tissues studied. Nevertheless, throughout the period of observation, C^{14} from cortisol-4-C^{14} disappeared from the spleen in a fashion corresponding closely to plasma, liver, and kidney (Fig. 2, Table 1).

Thymus

Thymus was studied only in the control group and in the group 14 days on a vitamin C deficient diet. The total amount of C^{14} persisted at about

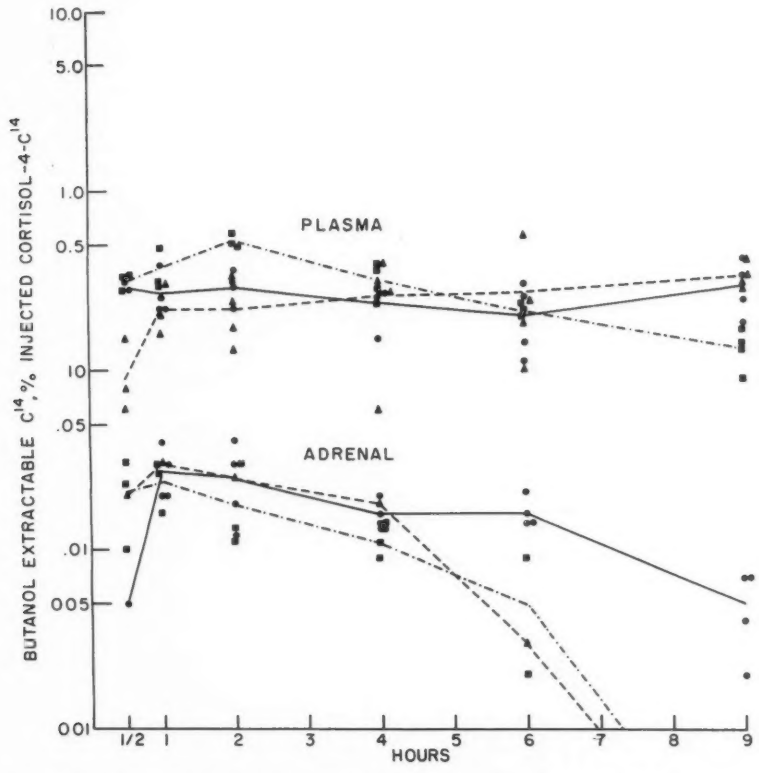

FIG. 4. Butanol extractable C^{14} from plasma* and adrenal following the intravenous administration of cortisol-4-C^{14} into normal animals and animals on vitamin C deficient diet for 7 and 14 days. The amounts of activity are expressed in % of injected cortisol-4-C^{14} and plotted semilogarithmically against time after injection.

●——● average curve from normal animals.
▲- - - - -▲ average curve from animals 7 days on vitamin C deficient diet.
■-·-·-·-·-■ average curve from animals 14 days on vitamin C deficient diet.

* The plasma values of normal animals and animals 7 days on a vitamin C deficient diet have already been published (6).

the same concentration from the first to the ninth hour after cortisol-4-C^{14} administration in all groups of animals. In normal animals all activity was recovered in methylene dichloride and in butanol before acid hydrolysis (Fig. 2, Table 1).

DISCUSSION

The present study indicated that cortisol and its metabolites may recycle through the adrenal gland. During the first four hours following the injection of cortisol-4-C^{14} the adrenal of the normal animal possessed the highest C^{14} concentration of all tissues studied, and unlike the C^{14} concentration in liver, kidney, and spleen, the C^{14} in the adrenal did not parallel the plasma values. In contrast to this relative retention of cortisol and its

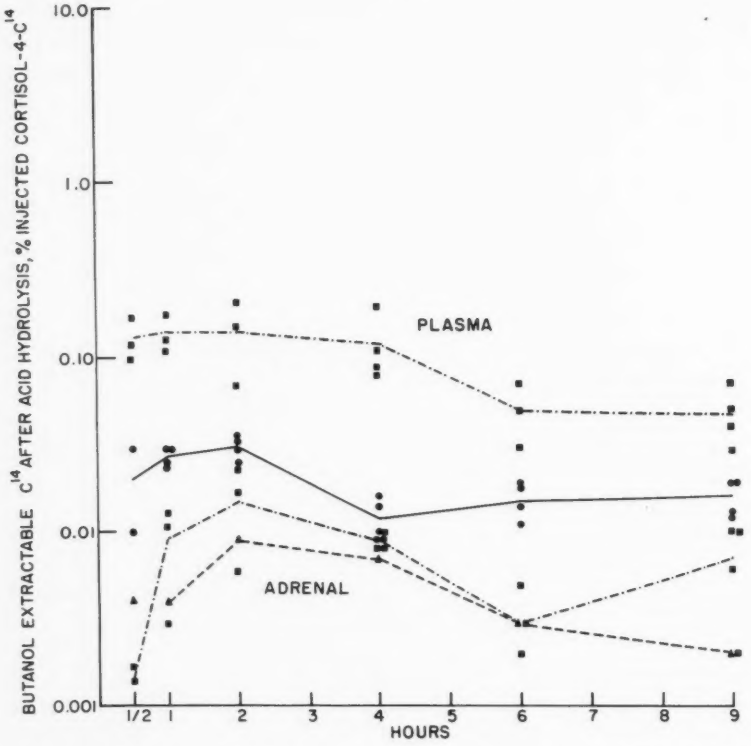

FIG. 5. Butanol extractable C^{14} after acid hydrolysis of plasma and adrenal following the intravenous administration of cortisol-4-C^{14} into normal animals and animals on vitamin C deficient diet for 7 and 14 days. The amounts of activity are expressed in % of injected cortisol-4-C^{14} and plotted semilogarithmically against time after injection.

●——● average curve from normal animals.
▲- - - - -▲ average curve from animals 7 days on vitamin C deficient diet.
■-·-·-·-·-■ average curve from animals 14 days on vitamin C deficient diet.

metabolites by the adrenal of the normal animal, guinea pigs with mild to moderate ascorbic acid deficiency showed a disappearance of C^{14} from the adrenal that was faster than from the other tissues. The vitamin C deficient animals, however, exhibited a clearance of C^{14} from the liver, kidney, spleen, and plasma like normal guinea pigs. This unique effect of ascorbic acid deficiency upon the adrenal could support the concept that the high concentration of ascorbic acid found in normal adrenals may inhibit the synthesis or release of adrenal steroids.

On the other hand cortisol-4-C^{14} may exchange with cortisol in the adrenal gland, with exogenous and endogenous cortisol and, perhaps, precursors forming a common metabolic pool. The rapid fall in C^{14} from the adrenals of vitamin C deficient animals seen 4–6 hours after the administration of cortisol could, therefore, indicate that less cortisol is being produced by the vitamin C deficient animals at that time than in the control group. This, however, is not very likely, since the ACTH response of vitamin C deficient animals would not indicate a reduction of adrenocortical capacity (6). The doses of cortisol injected in normal or vitamin C deficient animals were probably too low (0.0116μg./100 gm., 0.0464μg./100 gm.) to produce the classic inhibition of adrenal function (9). Thus, it is highly unlikely that the amount of cortisol given would inhibit the synthesis of adrenal steroids 4–6 hours after its administration.

The concentration of C^{14} in all tissues investigated equaled or exceeded that of the plasma (Table 1) and hence could not reflect the blood content of the tissues. Thus, the amount of C^{14} from cortisol-4-C^{14} in the adrenal and in the other tissues at any one time probably reflects the balance of entrance, exit, and metabolism of both exogenous and endogenous cortisol and its metabolites, and in the adrenal gland of the synthesis of endogenous cortisol as well.

Few data are available regarding metabolites of cortisol in plasma and in tissues of the guinea pig. It has been reported (10, 11, 12) that little or no 17-OHCS are conjugated as glucuronides but other types of conjugation have not been excluded. Non-conjugated polar 17-OHCS have been found in guinea pig urine (13, 14, 15). The low concentration of C^{14} from cortisol-4-C^{14} in the extracts of tissues and plasma did not permit a conclusive identification of the labeled material in the present study. However, besides cortisol, three major labeled steroids were found, one less polar and two more polar than cortisol, corresponding chromatographically to cortisone, 6β OH-cortisol, and 2α OH-cortisol, respectively (16). These metabolites have already been isolated from guinea pig urine (11, 13, 14, 15).

REFERENCES

1. Sayers, M. A., G. Sayers and L. Woodbury: *Endocrinology* **42**: 379. 1948.
2. Slusher, M. A. and S. Roberts: *Endocrinology* **61**: 98. 1957.
3. Oesterling, M. J. and C. N. H. Long: *Science* **113**: 241. 1951.

4. Done, A. K., R. S. Ely, L. R. Heiselt and V. C. Kelley: *Proc. Soc. Exper. Biol. & Med.* **83:** 722. 1953.
5. Hayano, M., N. Saba, R. I. Dorfman and O. Hechter: *Recent Progress in Hormone Research* **12:** 79. 1956.
6. Jones, R. S., L. Perič-Golia and K. Eik-Nes: *Endocrinology* **63:** 659. 1958.
7. Rinehart, J. F. and S. R. Mettier: *Am. J. Path.* **10:** 61. 1934.
8. Jones, R. S., Y. C. Mayne and K. Eik-Nes: *Endocrinology* **62:** 843. 1958.
9. Ingle, D. J., G. M. Higgins and E. C. Kendall: *Anat. Records* **71:** 363. 1938.
10. Wyngaarden, J. B., R. E. Peterson and A. R. Wolf: *J. Biol. Chem.* **212:** 936. 1955.
11. Péron, F. G. and R. I. Dorfman: *Endocrinology* **62:** 1. 1958.
12. Cohen, H. and W. Kleinberg: *Proc. Soc. Exper. Biol. & Med.* **100:** 375. 1959.
13. Burstein, S. and R. I. Dorfman: *J. Biol. Chem.* **206:** 607. 1954.
14. Burstein, S.: *J. Am. Chem. Soc.* **78:** 1769. 1955.
15. Nadel, E. M., B. G. Young and A. G. Hilgar: *Am. J. Physiol.* **196:** 273. 1959.
16. Perič-Golia, L., K. Eik-Nes and R. S. Jones: *Fed. Proc.* **18:** 499. 1959.

THE CATABOLISM OF ADRENOCORTICOTROPIC HORMONE: THE STABILITY OF ADRENOCORTICOTROPIC HORMONE IN BLOOD, PLASMA, SERUM, AND SALINE[1]

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ABSTRACT

Provisional USP Corticotropin (porcine ACTH) is inactivated by dog blood, plasma, and serum at 37° C in 3–4 hours. This ACTH is stable in 0.9% sodium chloride over a temperature range of 20–37° C and a pH range of 2–7 for at least 5–6 hours. It is also generally stable in 0.9% sodium chloride for at least one hour at 100° C at a pH of 1–2 but some experiments suggested slight reduction of activity under these conditions. Endogenous ACTH activity in human plasma is destroyed at 37° C but appears to be stable for at least 5–6 months in the frozen state.

THE fate of secreted ACTH has not been well defined. A number of observations strongly point to a prompt inactivation or excretion of ACTH activity. First, in man and in animals, a given amount of ACTH, administered continuously over a period of time, produces a greater biological effect than the same amount of ACTH administered as single or intermittent doses (1–4). Second, the duration of ACTH activity in the blood compartment of animals has generally been very short for endogenous ACTH (5, 6) exogenous species-specific ACTH (7, 8), and exogenous heterologous ACTH (9–11). Two observations in man have shown that exogenous heterologous ACTH activity quickly disappears from the blood (12, 13).

At least three possible modes of ACTH degradation exist. First, ACTH may be degraded in the process of exerting its influence on other tissues, notably ascorbic acid depletion and steroidogenesis in the adrenal cortex. To our knowledge, this possibility has never been shown. Second, it may be excreted from the body in sweat, stool, or urine. Evidence for these routes of removal exists only for urine and to date has been equivocal.

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Some investigators have not found ACTH activity in the urine of animals (8) and man (12, 14), but a few positive reports have appeared (15, 16). The basis of these conflicting reports may be that filtered or secreted ACTH is inactivated by urine during its passage from the tubule of the kidney to the exterior since, if the pH is not sufficiently low, rat urine will rapidly inactivate rat ACTH (8). Third, ACTH may be bound and inactivated by tissues in general or by some in particular. It has been shown that rat ACTH is bound by rat tissues, notably kidney and adrenal (8). Similar results have been observed by other investigators who injected I^{131} labeled ACTH into the rat (17, 18). Other investigations (19, 20) have shown that ACTH was inactivated when incubated *in vitro* with liver, kidney slices, diaphragm, and homogenates of liver, kidney, and adrenal. Since some inactivating capacity of these tissues remained after boiling it was concluded that the mechanism of inactivation was primarily one of binding or absorption. That commercial ACTH can be inactivated by plasma (21, 22) or blood (22, 23) has been reported, but such a process has not been found by others (6, 8) for rat ACTH in rat plasma and for sheep ACTH in rat plasma (19). Evidence has also been presented that human plasma to a slight extent alone, but to a more marked extent if activated with streptokinase, will degrade corticotropin A into trichloroacetic acid soluble peptides (24). Since the evidence for the last possibility (i.e., tissue and blood inactivation) appeared controversial, particularly with respect to the inactivation of ACTH by blood, it seemed important to re-examine the problem with a somewhat different approach.

GENERAL MATERIALS AND METHODS

Unless otherwise indicated, the ACTH used in this investigation was the USP Provisional Corticotropin Standard (porcine). Fresh blood, plasma, or serum was obtained from various donor dogs anesthetized with sodium pentobarbital given intravenously. Hydrogen ion concentrations of solutions were determined by the Beckman pH meter, Model G.

ACTH activity was assayed in the hypophysectomized dog preparation of Nelson and Hume (25). In this assay the standard or unknown material is injected into the femoral vein over a period of two minutes. After an interval of one minute adrenal venous blood is collected for the subsequent 10 minutes. The logarithm of the injected dose of ACTH (between 1 and 10 milliunits) bears a linear relationship to the total 10 minute output of 17-hydroxycorticosteroids (17-OHCS) in the adrenal venous effluent as measured by the method of Nelson and Samuels (25, 26). Since adrenal response could be determined every 35–40 minutes, changes in the ACTH activity of an incubated solution could be followed throughout a day using a single assay animal.

SPECIFIC METHODS AND RESULTS

1. *The in vivo binding or inactivation of ACTH by different tissues in the dog*

As a first step in studying the fate of circulating ACTH, several experiments were done to determine whether any tissue in the dog would preferentially remove ACTH from the blood stream.

The hypophysectomized dog preparation for ACTH assay (25) with cannulae in one femoral vein, and one adrenal vein, was used. By means of polyethylene cannulae in the splenic vein, a femoral artery, a renal artery, and in the adrenal artery (contralateral to the adrenal gland with the venous cannula) it was possible to inject standard amounts of ACTH through liver, rear leg, kidney, and adrenal, and to compare the steroidogenic effect (i.e., in adrenal venous effluent) with standard ACTH injected into the femoral vein (which would bypass any tissue except lung on the first circulation).

In a given dog preparation the average steroidogenic response after a femoral vein injection of ACTH was taken as 100% and the average response following injection through a tissue was expressed as a per cent of the average femoral vein response. In a series of six dogs, the following results were obtained (mean ± one standard deviation):

Liver	92% ± 21	(N = 12)
Leg	87% ± 7	(N = 6)
Kidney	85% ± 26	(N = 12)
Adrenal Gland	84% ± 13	(N = 12)

Thus, no preferential binding of ACTH by any of the tissues studied was demonstrable in these experiments.

2. *ACTH inactivation in heparinized dog blood at 37° C*

In the following experiments, ACTH (as a solution of 10 mU. (milliunits) per ml. in 0.9% NaCl made 0.01N with respect to HCl) was added to heparinized dog blood to give a final mixture concentration of 0.25, 0.5 or 1.0 mU. of ACTH per ml. The resulting mixtures had a "pH" of 7.5 (in room air).

Immediately after mixing the ACTH and blood (zero time) an amount of the mixture calculated to contain 5 mU. of added ACTH was assayed. The remainder of the mixture was placed in a 37° C water bath and reassayed at intervals alternating with 5 mU. of ACTH standard (made up in concentrations of 2.5, 5.0 or 10 mU. per ml. in 0.9% NaCl made 0.01N to HCl). In one experiment 2.5 mU. doses of ACTH instead of 5 mU. were assayed. The timing of an individual response was that of the start of the injection of standard or blood mixture after the mixing (zero time) of blood and ACTH. The standard ACTH solution (pH = 2) was kept at room temperature (subsequent data will show that there was no difference during a day in such ACTH standards whether stored at 20 or 37° C, nor at pH 2 or 7.5). Control (nothing injected) blood collections were made at the beginning and the end of an experimental day. The response to a standard dose of ACTH was also determined prior to the timed incubation period.

Five such experiments were done. The detailed data of a typical experiment are presented in Figure 1. This experiment demonstrates that while

the responsivity of the preparation to a constant dose of ACTH standard had declined only slightly after three hours, the ACTH activity of the blood mixture had reached a non-detectable (i.e., control) level very quickly.

In Table 1, all five experiments are recorded. For any individual experiment it can be seen that the responsivity of the dog to a constant ACTH dose generally declined moderately through the experimental day, though in experiment #5 the dog appeared to become more responsive. On the

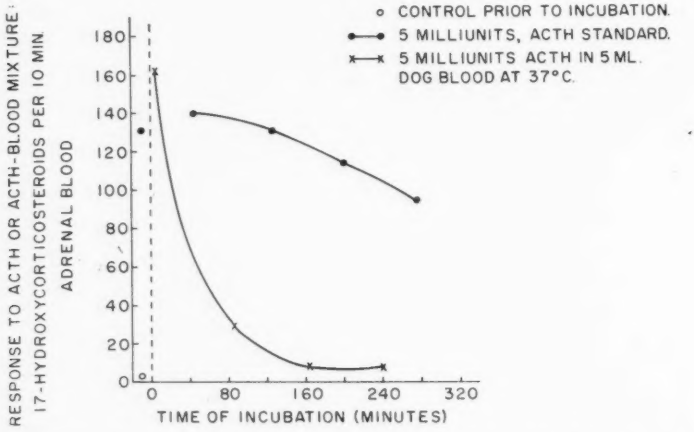

FIG. 1. Inactivation of porcine ACTH in heparinized dog blood *in vitro* at 37° C. Standard ACTH and ACTH incubated in dog blood were injected alternately into the same assay animal for estimation of remaining ACTH activity.

other hand, it is apparent in all five experiments that the adrenal response to the ACTH-blood mixture declined at much more rapid rates, reaching control levels in three hours or less.

The results of each experiment are summarized at the bottom of Table 1 where the percentage fall in a column is calculated in terms of the original and final figure in the column. In each experiment the fall in the ACTH activity (steroidogenic) of the ACTH-blood mixture was always much greater than that of the standard.

While there are some small variations in these results, they strongly suggest that dog blood at 37° C is capable of inactivating added Provisional USP Corticotropin Standard (porcine).

3. *ACTH inactivation in dog plasma or serum at 37° C*

The following experiments were carried out in the same manner as experiments 1–5 described above. Pertinent differences are pointed out. The data are presented in Table 2.

(a) Since blood appeared to inactivate ACTH, heparinized plasma was examined in two experiments to see if it were the site of this process. The results are recorded in Table 2 as experiments #6 and #7. Experiment #6 is also recorded in Figure 2. In experiment #6, while the responsivity of the dog to standard ACTH tended to increase, there was a fall in the ACTH activity of the ACTH-plasma mixture to control values within four hours. In experiment #7, the rate of decline in ACTH activity of the plasma-ACTH mixture was slower.

TABLE 1. INACTIVATION OF ACTH IN BLOOD

Incubation time in minutes	Assay animal									
	1		2		3		4		5	
	Milliunits of ACTH standard or ACTH-blood mixture injected									
	5 std.	5 10 ml. blood	5 std.	5 10 ml. blood	2.5 std.	2.5 10 ml. blood	5 std.	5 5 ml. blood	5 std.	5 10 ml. blood
	17-OHCS response in adrenal venous blood, μg./10 min.									
Before incubation	0*		3*		0* 80†		3* 131†		0* 112†	
5– 10		42		160	102	62		162		
45– 50	44		117				140			196
80– 85		41		117		11		29		93
117	31									39
126			179				131			
137					105					
155–160		6							149	
163								8		
170–175				4		9				
190–195	27									4
200–205			63				114			
213					76					
224									173	
% fall from original value	39	86	61	98	5	85	13	95	0	98

* Control (nothing injected).
† Standard ACTH injected.

(b) To rule out an ACTH inactivating contaminant peculiar to the Provisional USP Corticotropin Standard, two experiments were carried out by incubating porcine ACTH preparations from different sources with heparinized plasma. In experiment #8, corticotropin injection (porcine ACTH) prepared by the Upjohn Company (Lot KF 038FA) was used. For experiment #9, a purified ACTH preparation assaying 80 USP units per mg. was kindly supplied to us by Dr. E. B. Astwood. The latter ACTH was diluted in 0.9% NaCl, made 0.01N to HCl, and containing 200 mg. per cent of gelatin.

In experiment #8, prior to the timed incubation period, there was no response to the injections of either the ACTH solvent or fresh dog plasma

(1 and 0 respectively, omitted from Table 2). In experiment #9, there was a rather high basal output of 17-hydroxycorticosteroids in the adrenal venous effluent (36 μg. 17-OHCS/10 minutes) which is reflected in the apparent response to ACTH solvent (55 μg. 17-OHCS/10 minutes, omitted from Table 2), and the rather high response to the standard ACTH injection (210 μg. 17-OHCS/10 minutes). These figures probably mean that a remnant of anterior pituitary, not removed, remained viable during this period. The low response to plain plasma (9 μg. 17-OHCS/10 minutes, omitted from Table 2), just before the incubation period, indicated that the endogenous ACTH production of the preparation had fallen to negligible levels prior to the beginning of the incubation. In both experiments #8 and #9, it is again apparent that there is a decline in the ACTH activity of the incubation mixture reaching control levels in 3-4 hours.

TABLE 2. INACTIVATION OF ACTH IN PLASMA AND SERUM

Incubation time in minutes	Assay animal											
	6		7		8		9		10		11	
	Milliunits of ACTH standard or ACTH-plasma (or serum) mixture injected											
	2.5 std.	2.5 5 ml. plasma	5 std.	5 5 ml. plasma	3 std.	3 6 ml. plasma	5 std.	5 10 ml. plasma	2.5 std.	2.5 5 ml. plasma	5 std.	5 10 ml. serum
	17-OHCS response in adrenal venous blood, μg./10 min.											
Before incubation	5* 119		16* 86		1* 230		36* 210		3* 87		0* 79	
0– 5				89		261		153		73		81
40– 45		109	91			244		132		34		65
75– 80	133			88	368		173		54		83	
110–120		33	117			91		37		9		11
150–155		29		42	255		165			5	82	
185–195	181		120			27		14	31			10
220–230		4		55	267		82					
232											64	
259–263	182		109									
% fall from original value	0	96	0	38	0	90	61	91	64	93	19	88

* Control (nothing injected).

(c) Some evidence has suggested (27) that heparin may be a physiological inhibitor of ACTH but others (21, 28) have failed to substantiate these observations. To rule out the effect of heparin, experiment #10 was carried out with oxalated plasma and Provisional USP Corticotropin Standard, and again the ACTH activity in the incubation mixture declined to control levels in 2–3 hours as seen in Table 2. A plain plasma control injection (omitted from Table 2), just before the incubation period, gave a response of 11 μg. 17-OHCS/10 minutes.

(d) Experiment #11 was similar to experiment #9 except that the ACTH was incubated with fresh serum (instead of heparinized plasma) from another dog. The standard ACTH (in a concentration of 1 mU. per ml. of 0.9% NaCl, made 0.01N to HCl, and containing 200 mg. per cent of gelatin) was also incubated at 37° C along with the ACTH-serum mixture.

It is apparent that serum is able to inactivate a highly purified hog ACTH. Injections of ACTH solvent (0.01N HCl in 0.9% NaCl containing 200 mg. per cent gelatin) and plain serum prior to the incubation period gave responses of 1 and 0 μg. 17-OHCS/10 minutes respectively, but are omitted from Table 2 for simplicity.

The results of each experiment are summarized at the bottom of Table 2 where the percentage fall in a column is calculated in terms of the original and final figure in the column. In each experiment the fall in the ACTH activity (steroidogenic) of the ACTH-plasma or serum mixture was always greater than that of the standard ACTH solution.

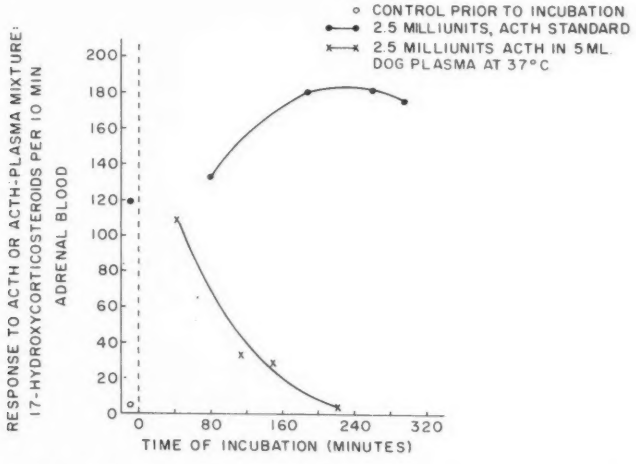

FIG. 2. Inactivation of porcine ACTH in heparinized dog plasma at 37° C. Standard ACTH and ACTH incubated in dog plasma were injected alternately into the same assay animal for estimation of remaining ACTH activity.

4. *The inactivation of endogenous ACTH by human plasma*

(a) To determine whether there was a significant degree of inactivation of endogenous ACTH in the plasma of humans, advantage was taken of a patient, C.R. (PBBH 9G418) reported elsewhere (29), who had a tumor of the pituitary gland which was secreting large amounts of ACTH. Heparinized plasma was freshly drawn. A 2 ml. aliquot was assayed in the hypophysectomized dog and the remainder was incubated at 37° C. Two ml. aliquots were reassayed at intervals. Provisional USP Corticotropin Standard (freshly dissolved in 0.9% NaCl, made 0.01N to HCl, and stored at room temperature during the experiment) was injected at intervals, at two dose levels (2 and 8 mU.). The results of this study are presented in Figure 3. It can be readily seen that while the responsivity of the dog to 2

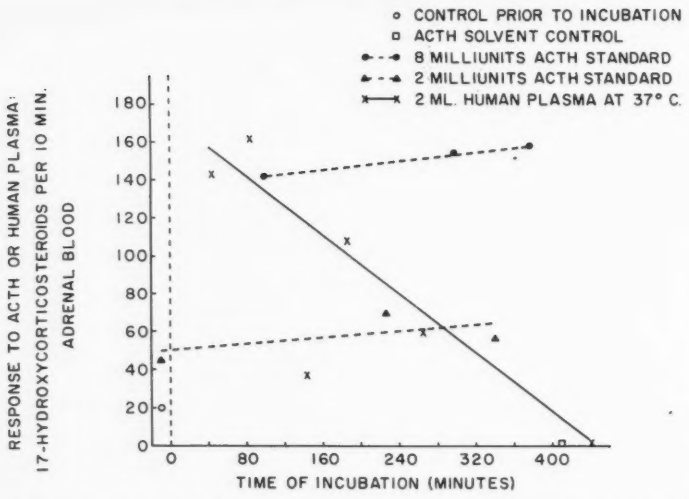

FIG. 3. Inactivation of endogenous ACTH by human plasma. Standard ACTH (at two dose levels) and human plasma, incubated *in vitro* at 37° C, were injected alternately into the same assay animal for estimation of remaining ACTH activity.

and 8 mU. of standard ACTH remained constant during the experiment, the ACTH activity of the patient's plasma steadily declined reaching undetectable levels in seven hours with an approximate half-life of 190 minutes.

(b) In hopes of corroborating the above observations, portions of heparinized plasma specimens from the same patient (C.R.) obtained $5\frac{1}{2}$ and 6 months earlier on different days were assayed for ACTH activity. The remaining portions were incubated at 37° C, and reassayed after 77 to 225 minutes. In the first specimen (Table 3), it is apparent that even after apparent stability for $6\frac{1}{2}$ months in the frozen state there was inactivation of ACTH demonstrable after incubation at 37° C for 225 minutes. In the next two specimens from C.R., after 5 months in the frozen state, there was no demonstrable inactivation of ACTH when incubated at 37° C for 77–156 minutes.

TABLE 3. INACTIVATION OF ENDOGENOUS ACTH BY HEPARINIZED HUMAN PLASMA

Patient	Plasma ACTH mμ/100 ml. before freezing	Time plasma frozen	Plasma ACTH activity, milliunits/100 ml.		Time (min.) incubation 37° C
			Before incubation	After incubation	
C.R.	43	6.5 mos.	34	<10	225
C.R.		5 mos.	216*	294*	77
C.R.	409	5 mos.	393*	342*	156
T.M.		156 min.	54	14	219
T.M.		100 min.	59	12	184

* Difference not considered significant

A second patient (T.M., PBBH 3M357), who on clinical and chemical evidence was felt to have the same pathological process as C.R., presented another opportunity to study the inactivation of endogenous ACTH by human plasma. In both experiments fresh samples of plasma were assayed before and after incubation at 37° C for 184 and 219 minutes. In both experiments (4th and 5th lines of Table 3) there appeared to be a significant decline in ACTH activity.

(c) The first specimen of plasma from C.R., assaying 34 mU. per 100 ml. in Table 3, had previously been assayed 6½ months earlier and had been found to have an ACTH activity of 43 mU. per 100 ml. plasma. Similarly, the third specimen of C.R., in Table 3 assaying 393 mU. per 100 ml., had 5 months earlier been found to have an ACTH activity of 409 mU. per 100 ml. plasma.

It appears that human plasma can inactivate human ACTH at 37° C *in vitro*, though this capacity may decline on storage in the cold. It is also of interest that no significant decline in ACTH activity of frozen plasma occurred over 5–6½ months.

5. *Inactivation of heated ACTH by plasma*

It has been reported that rat plasma does not inactivate rat ACTH if the ACTH is "boiled" at a pH of 7.5 for 2–3 hours during its extraction (30), or at a pH of 1 prior to incubation (8). In many of the experiments in this study, demonstrating ACTH inactivation in blood, plasma, or serum, Provisional USP Corticotropin was used. This ACTH preparation is relatively impure and could possibly have contained peptidases and proteases which have been shown to occur in crude anterior pituitary preparations (31), and which could possibly cause inactivation when ACTH is added to *in vitro* systems (19). Therefore, the following experiments were done.

(a) In two experiments Provisional USP Corticotropin was made up to a concentration of 10 mU. per ml. of 0.9% NaCl made 0.1N to HCl. An aliquot was diluted with 0.9% NaCl to a concentration of 1 mU. per ml. at pH 2.0 and kept at room temperature as unheated ACTH standard. The remainder was heated to 100° C in a water bath for one hour. An aliquot of the heated ACTH was similarly diluted with 0.9% NaCl to a concentration of 1 mU. per ml. at a pH of 2 and kept at room temperature as the heated ACTH standard. A second aliquot of the heated ACTH was added to fresh heparinized dog plasma to a final concentration of 0.5 mU. per ml. and, together with the two standard ACTH solutions, was incubated at 37°C.

Using the hypophysectomized dog preparation the responses to aliquots of the plasma-heated ACTH mixture, and to aliquots of the heated and unheated ACTH standard solutions (all estimated to contain the same amount of original ACTH activity) were determined at intervals.

Figure 4 shows the results of one experiment. No response was observed when nothing, ACTH diluent, or fresh heparinized dog plasma was injected. There was no significant difference in the response to heated or unheated ACTH standard solutions. While the responses to the standard heated ACTH did not fall significantly below the initial response during the experiment, it is apparent that the dog plasma inactivated the added ACTH (previously heated to 100° C for 1 hour at a pH of 1). The pH of the ACTH standard solutions was 2 and that of the ACTH-plasma mixture was 7.7 throughout the experiment.

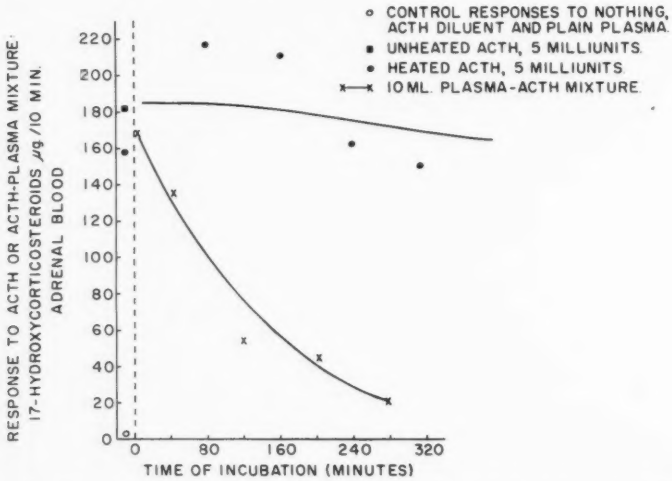

FIG. 4. Inactivation of porcine ACTH (heated to 100° C for 1 hour in 0.9% NaCl-0.1N HCl) by dog plasma. Responses are shown in terms of 17-hydroxycorticosteroids per 10 minutes in the adrenal venous blood of the same hypophysectomized dog to the sequential intravenous injection of control solutions, ACTH standards, and plasma-ACTH mixtures for the estimation of remaining ACTH activity.

A second identical experiment confirmed this result in which ACTH activity in the plasma-heated ACTH mixture became non-detectable even more quickly in 196 minutes.

(b) Two further experiments were done which were essentially the same as the above except that the ACTH was heated to 100° C in 0.01N HCl for 1 hour prior to addition to plasma. In both of these experiments inactivation of the heated ACTH occurred. ACTH activity in the heated ACTH-plasma mixtures became non-detectable in 232 and 233 minutes.

6. *The Stability of ACTH at 20 and 37° C*

In early experiments ACTH standard solutions were kept at room temperature and in later experiments they were incubated at 37° C along with

the plasma-ACTH mixtures under study. Two experiments were done to determine whether there was a difference in the stability of standard ACTH solutions when incubated over the temperature range of 20–37° C.

One experiment was performed in which Provisional USP Corticotropin was dissolved to a concentration of 10 mu. per ml. in 0.9% NaCl (adjusted to pH 1 with HCl). A portion was first heated to 100° C for 1 hour. Then heated and unheated ACTH solutions were diluted with 0.9% NaCl to give a concentration of 1 mu. per ml. and a pH of 2. A portion of each was kept

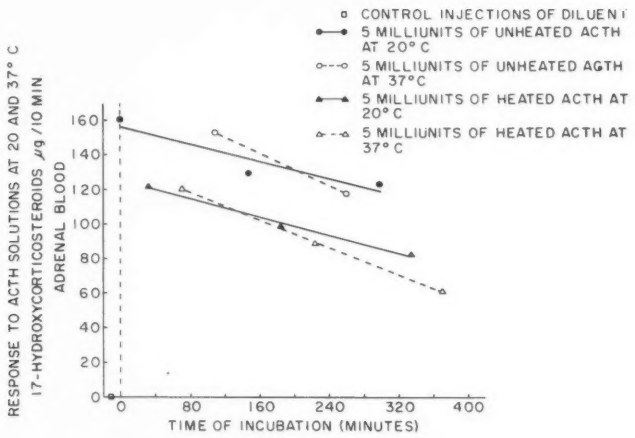

FIG. 5. Equal portions heated and unheated ACTH (in 0.9% NaCl and at a pH of 2) at 20° C and 37° C were alternately injected intravenously into the hypophysectomized dog to determine differences in remaining ACTH activity during a period of 5 hours.

at 20° C and another at 37° C. Each portion was alternatively assayed over a period of six hours. The results are presented in Figure 5. Control injections of ACTH diluent produced no 17-OHCS response in this dog. The responsivity of the dog preparation declined slowly but equally to both ACTH solutions, although the ACTH, which had been heated to 100° C, gave consistently lower responses suggesting some destruction at 100°C and a pH of 1. The difference is sufficiently small to have been a dilution error.

A second experiment was identical except that the ACTH was not heated to 100° C prior to the incubation period. Over a period of five hours no difference in potency was observed between the solutions at 20° C and 37° C. Therefore, it was concluded that this ACTH in 0.9% NaCl and at a pH of 2 was equally stable at temperatures of 20° C–37° C incubated for at least a period of 5–6 hours.

7. The stability of ACTH at different hydrogen ion concentrations

In all experiments reported in this study, unless otherwise noted, the pH of the ACTH standard solutions was 2.0 and that of the plasma-ACTH

mixtures was 7–8. It was desirable to determine how much the pH difference contributed to the apparent stability of the standard solutions (20–37° C) as compared with the progressive inactivation of the plasma-ACTH mixtures (37° C).

In one experiment Provisional USP Corticotropin Standard was dissolved to a concentration of 10 mu. per ml. in 0.9% NaCl, adjusted to pH 1 with HCl, and heated to 100° C for 1 hour. It was then diluted with 0.9% NaCl to a concentration of 1 mu. per ml. and a pH of 2. A portion of the latter was adjusted to a pH of 7.8 with sodium hydroxide. Both diluted

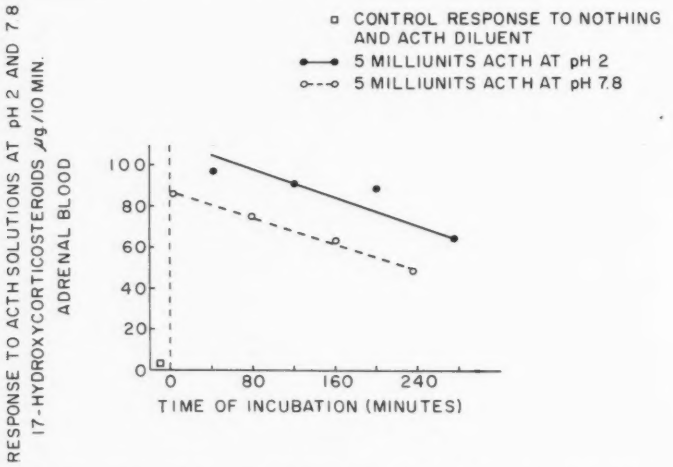

FIG. 6. Equal portions of ACTH solution at a pH of 2 or a pH of 7–7.8 were alternately injected into the hypophysectomized dog to determine differences in ACTH activity during a period of $4\frac{1}{2}$ hours.

standard solutions were incubated at 37° C and the activity alternately determined in the dog preparation. The results are presented in Figure 6. The absolute activity of the standard at pH 7.8 (this had fallen to pH 7.0 after 6 hours) was initially about 20 per cent lower than the standard at pH 2.0. During the subsequent $5\frac{1}{2}$ hour experimental period, there was no evidence that the activity of the ACTH solution at a pH value of 7.8–7.0 declined more rapidly than that at pH 2.0.

A second experiment was carried out as in Section 5 (a) in which heated ACTH was added to plasma to give a final pH of 7.3. The standard ACTH was adjusted with sodium hydroxide to a similar pH (7.6). The observed responses to subsequent injections of the ACTH standard solution declined during the day indicating either inactivation or decreasing sensitivity of the dog. The activity of the plasma-ACTH mixture on the other hand declined much more rapidly. These two experiments indicate that at 37° C there is no difference in the rate of decline in activity of ACTH in 0.9%

NaCl over the pH range of 2 to 8 in 6 hours, and that the pH of the plasma-ACTH mixtures was not primarily responsible for the inactivation of ACTH observed in this system.

DISCUSSION

The experiments demonstrate the inactivation of porcine ACTH in dog blood, plasma, and serum. The significance of this observation has been given support by the demonstration of endogenous ACTH inactivation in human plasma *in vitro*. These observations are in agreement with the observations of others (21, 22, 24), but not with those of Sayers and coworkers (6, 8) nor Geschwind and Li (19). Geschwind and Li (19) may not have observed inactivation since ACTH was added to plasma in very large amounts giving concentrations in the order of 1 unit/ml. Sydnor and Sayers (6) incubated the plasma-ACTH system for only 1 minute and hence an inactivating system might not be apparent. However, the reason why Richards and Sayers (8) did not observe inactivation when rat ACTH was added to rat blood in a concentration of 2–8 mU. per ml. at 37° C for 1 hour is not apparent. Other reasons for these discrepancies are not immediately apparent but possibilities will be discussed when the mechanism of inactivation is considered in another paper (32).

The importance of this inactivating system in the blood in the regulation of ACTH blood levels remains to be determined. However, in view of the above observations it may be pointed out that experiments demonstrating disappearance of ACTH activity from the vascular compartment do not necessarily indicate excretion nor binding of ACTH to tissue but may mean inactivation in blood itself.

On the basis of these observations it would appear that in the collection of human blood, plasma, or serum specimens for the assay of ACTH activity, care should be taken to cool and freeze the specimen as quickly as possible.

Acknowledgments

We are indebted to Dr. George W. Thorn for his interest and advice in the course of this investigation.

The technical assistance of Mrs. N. Meegan, Miss N. Madeira, and Mrs. E. Kolar was very much appreciated.

REFERENCES

1. Ingle, D. J.: Symposium of Pituitary-Adrenal Function, 1949, p. 49. The American Association for the Advancement of Science. 1950.
2. Ingle, D. J.: Adrenal Cortex, Transactions of the Second Conference, 1950, p. 42. Sponsored by Josiah Macy, Jr. Foundation, ed. Ralli, E. P. New York. 1951.
3. Nelson, D. H., L. T. Samuels, D. G. Willardson and F. H. Tyler: *J. Clin. Endocrinol.* **11**: 1021. 1951.
4. Renold, A. E., D. Jenkins, P. H. Forsham and G. W. Thorn: *J. Clin. Endocrinol. and Metab.* **12**: 763. 1952.

5. Van Dyke, D. C., M. E. Simpson, C. H. Li and H. M. Evans: *Am. J. Physiol.* **163:** 297. 1950.
6. Sydnor, K. L. and G. Sayers: *Proc. Soc. Exper. Biol. and Med.* **83:** 729. 1953.
7. Richards, J. B., M. Merkin, C. P. Cheng and G. Sayers: *J. Clin. Endocrinol.* **10:** 809. 1950.
8. Richards, J. B. and G. Sayers: *Proc. Soc. Exper. Biol. and Med.* **77:** 87. 1951.
9. Greenspan, F. S., C. H. Li and H. M. Evans: *Endocrinology* **46:** 261. 1950.
10. Gemzell, C. A., D. C. Van Dyke, C. A. Tobias and H. M. Evans: *Endocrinology* **49:** 325. 1951.
11. Cats, A. and A. A. H. Kassenaar: *Acta Endocrinol.* **24:** 43. 1957.
12. Sayers, G. T., T. W. Burns, E. H. Tyler, B. V. Jager, T. B. Schwartz, E. S. Smith, L. T. Samuels and H. W. Davenport: *J. Clin. Endocrinol.* **9:** 593. 1949.
13. Meakin, J. W., J. E. Bethune, R. H. Despointes and D. H. Nelson: In preparation.
14. Locke, W., A. Albert and E. J. Kepler: *Proc. Soc. Exper. Biol. and Med.* **72:** 470. 1949.
15. Rubin, B. L., Dorfman, R. I. and Dorfman, A.: *J. Clin. Endocrinol. and Metab.* **14:** 154. 1954.
16. Fujita, T.: *J. Clin. Endocrinol. and Metab.* **17:** 512. 1957.
17. Sonenberg, M., A. S. Keston and W. L. Money: *Endocrinology* **48:** 148. 1951.
18. Cats, A. and A. A. H. Kassenaar: *Acta Endocrinol.* **24:** 35. 1957.
19. Geschwind, I. I. and C. H. Li: *Endocrinology* **50:** 226. 1952.
20. Birmingham, M. K. and E. Kurlents: *Endocrinology* **62:** 47. 1958.
21. Reiss, M., F. W. Badrick, I. D. K. Halkerston and C. Plaice: *Nature* **168:** 206. 1951.
22. Pincus, G., T. F. Hopkins and O. Hechter: *Arch. Biochem.* **37:** 408. 1952.
23. Pincus, G.: Adrenal Cortex, Transaction of the Second Conference, 1950, p. 42. Sponsored by the Josiah Macy, Jr. Foundation. Ed. Ralli, E. P. New York. 1951.
24. Mirsky, I. A., G. Perisutti and N. C. Davis: *J. Clin. Investig.* **38:** 14. 1959.
25. Nelson, D. H. and D. M. Hume: *Endocrinology* **57:** 184. 1955.
26. Nelson, D. H. and L. T. Samuels: *J. Clin. Endocrinol. and Metab.* **12:** 519. 1952.
27. Godlowski, Z. Z.: *Brit. Med. J.* **1:** 854. 1951.
28. Fekete, G.: *Experientia.* **11:** 310. 1955.
29. Nelson, D. H., J. W. Meakin, J. B. Dealy, D. D. Matson, K. Emerson, Jr. and G. W. Thorn: *New England J. Med.* **259:** 161. 1958.
30. Li, C. H., M. E. Simpson and H. M. Evans: *J. Biol. Chem.* **149:** 413. 1943.
31. Adams, E. and Smith, E. L.: *J. Biol. Chem.* **191:** 651. 1951.
32. Meakin, J. W. and D. H. Nelson: *Endocrinology* **66:** 73. 1960.

THE CATABOLISM OF ADRENOCORTICOTROPIC HORMONE: SOME CHARACTERISTICS OF AN ACTH-INACTIVATING SYSTEM IN PLASMA[1]

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ABSTRACT

It has been previously shown that porcine ACTH is inactivated *in vitro* by canine blood, plasma, and serum, and that human plasma will inactivate endogenous ACTH.

In the present report it has been demonstrated that this ACTH inactivating system(s) is destroyed by heating to 60° C for one hour, that the rate of inactivation is temperature dependent, and that L-cysteine inhibits the process. These findings suggest that the ACTH inactivating system in plasma may be an enzymatic oxidation of the molecule to a biologically inactive form.

THE inactivation of porcine ACTH by canine blood, plasma, and serum, *in vitro* at 37° C, and a similar inactivation of endogenous ACTH by human plasma (1) prompted inquiry into the nature of the inactivating system. The relationship of the biological activity of ACTH to various chemical and enzymatic alterations in the structure of the molecule has been recently reviewed by Behrens and Bromer (2). It has been shown that enzymatic hydrolysis, oxidation by various oxidizing agents, exposure to strong alkali, iodination, and other chemical treatments alter structure and function to varying degrees.

In the following account, heat lability, the relation of temperature to rate of reaction, and the effect of L-cysteine on the ACTH inactivating system in plasma, were examined.

GENERAL MATERIALS AND METHODS

In the first two groups of experiments, USP Provisional Corticotropin Standard was used and in the third group of experiments dealing with L-cysteine effects, a more purified porcine ACTH assaying 80 units per mg. was used.[4] Fresh plasma was obtained from various donor dogs anesthetized with intravenously administered sodium pentobarbital. Hydrogen ion concentrations were determined by the Beckman pH meter, Model G.

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[2] Research Fellow in Medicine of the American College of Physicians (1957–58), and a Life Insurance Medical Research Fellow (1958–59).

[3] Investigator, Howard Hughes Medical Institute.

[4] Kindly supplied to us by Dr. E. B. Astwood.

The activity of ACTH added to canine plasma under the conditions of the three groups of experiments was followed throughout an experimental day by assaying aliquot of the incubation medium in one hypophysectomized dog preparation as described by Nelson and Hume (3). In this assay the standard or unknown material is injected into the femoral vein over a period of two minutes. After an interval of one minute, adrenal venous blood is collected for the subsequent 10 minutes. The logarithm of the injected dose of ACTH (between 1 and 10 mU.) bears a linear relationship to the total 10 minute output of 17-hydroxycorticosteroids (17-OHCS) in the adrenal venous effluent as measured by the method of Nelson and Samuels (4, 3). Since this procedure could be repeated every 35–40 minutes, changes in the ACTH activity of an incubated solution could be followed throughout a day using one assay animal.

SPECIFIC METHODS AND RESULTS

1. *Thermolability of the ACTH inactivating factor in dog plasma*

This was studied by adding ACTH (as a solution of 10 mU./ml. in 0.9% NaCl 0.1N HCl, heated previously to 100° C for one hour) to dog plasma which had been heated to 60° C for one hour. The final concentration of ACTH activity in the plasma medium was 0.5 mU. per ml. ACTH standard was also prepared from the same heated ACTH by diluting with 0.9% NaCl to give a solution of 1 mU. per ml. and a pH of 2. Both standard and ACTH-plasma mixture were then incubated at 37° C and the ACTH activity determined. The pH of the plasma-ACTH mixture remained constant through the experiments (7.4–7.9 for different experiments).

The results of this experiment are presented in Figure 1 and as experiment #1 in Table 1 from which it may be seen that no significant inactivation of the ACTH (added to the heated plasma) occurred, indicating heat

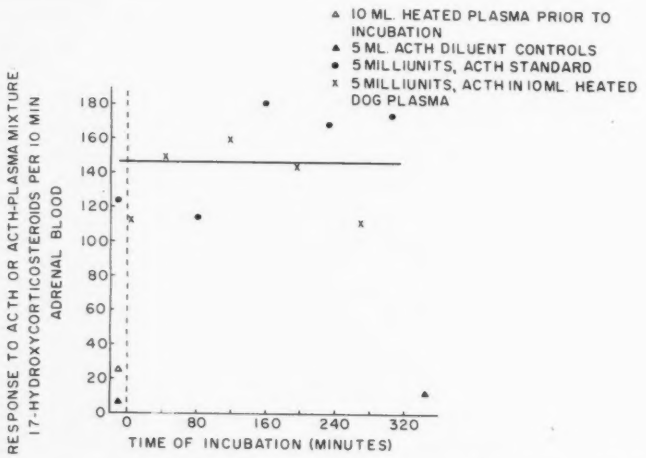

FIG. 1. No inactivation of porcine ACTH in heparinized, heated (60° C for 1 hour) dog plasma which with standard ACTH was alternately injected into the same assay animal for estimation of remaining ACTH activity.

TABLE 1. THERMOLABILITY OF THE ACTH INACTIVATING FACTOR IN DOG PLASMA

Incubation time in minutes	Assay animal							
	1		2		3		4	
	Milliunits of ACTH in standard or ACTH-plasma mixture injected							
	5 Std.	5 in 10 ml. heated plasma	5 Std.	5 in 10 ml. heated plasma	5 Std.	5 in 10 ml. heated plasma	5 Std.	5 in 10 ml. heated plasma
	17-OHCS response in adrenal blood, μg/10 minutes							
Before incubation	7* 124		5* 141		0* 65		3* 187	
3		112		147		51		48
42– 45		149		191		60		32
79– 83	114		157		96		116	
119–122		159		179		90		25
152–161	181		124		115		63	
189–196		144		151		62		
201								26
227–238	168		138		111		64	
264–273		111		180		51		
301–309	174		140		100			
% fall from original value	0	0	1	0	0	0	71	46

* Control (nothing injected).

liability of the inactivating system. In this experiment control injections of ACTH diluent, and simple heated plasma produced 17-OHCS responses of 6 and 25 μg./10 minutes respectively prior to the period of incubation. Experiment #2 of Table 1 gave essentially the same results as experiment #1.

In experiment #3, a control injection of plain heated plasma produced a 17-OHCS response of 3 μg./10 minutes prior to the incubation period. In this experiment the 17-OHCS responses to plasma-ACTH injections were consistently lower than responses to standard ACTH, but there was not a significant decline in response to the ACTH in plasma during incubation.

In experiment #4, control injections of ACTH diluent and plain heated plasma produced 17-OHCS responses of 1 and 2 μg./10 minutes respectively. In this experiment the responsivity of the assay animal declined during the experimental day, but there was no greater decrease in response to heated plasma-ACTH mixture than to standard ACTH. As in experiment #3, the responses to the heated plasma-ACTH mixture were always lower than to the standard.

These experiments demonstrate that canine plasma heated to 60° C for one hour loses its ability to inactivate porcine ACTH at 37° C as previously described (1). The lower but constant (relative to standard ACTH) 17-OHCS responses to the heated plasma-ACTH mixtures in experiments #3

and #4 are not readily explicable except on the basis of dilution error or an immediate physical binding of ACTH to the heated (partially denatured) plasma protein.

2. *The effect of temperature on the rate of inactivation of ACTH by plasma*

In one experiment ACTH was added to canine plasma. Half of this mixture was incubated at 0° C and half at 37° C. Standard ACTH was made up in 0.9% sodium chloride made 0.01N to HCl in a concentration of 1 mu. per ml. and was kept at 20° C. The results are presented in Figure 2 from which it may be seen that the activity of the mixture at 37° C declined rapidly whereas that at 0° C did not differ significantly from that of the standard ACTH.

3. *The effect of* L-*cysteine on the inactivation of ACTH by plasma*

Three experiments were done in which porcine ACTH was added (to give a final concentration of 0.5 mu. per ml.) to two portions of fresh dog plasma and incubated at 37° C. One of the portions of plasma also contained 1% (w/v) L-cysteine. Aliquots of each portion of plasma mixture, along with standard ACTH solutions, were alternately assayed for ACTH activity. The results are recorded in Table 2.

There were no significant changes in the pH of the plasma mixtures (7.2–8.0) during incubations in all three experiments. L-Cysteine plus plain plasma, and ACTH diluent (0.9% NaCl made 0.01N to HCl and 200 mg. per cent gelatin) gave 17-OHCS responses less than control (nothing in-

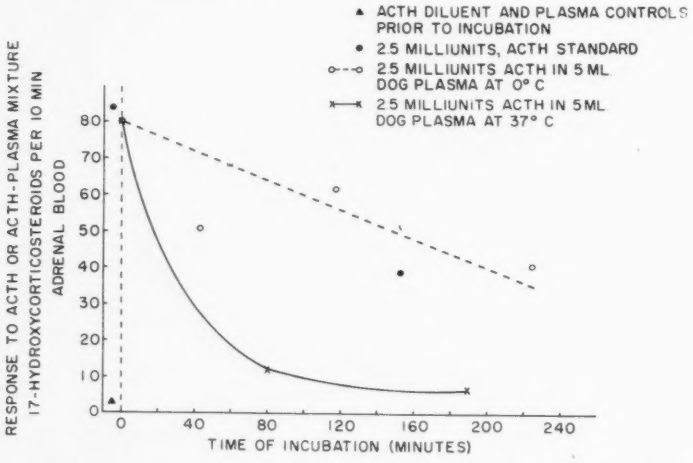

FIG. 2. No inactivation of porcine ACTH in dog plasma at 0° C. Standard ACTH, and ACTH incubated in dog blood at 0° C or 37° C were injected alternately into the same assay animal for estimation of remaining ACTH activity.

TABLE 2. THE EFFECT OF L-CYSTEINE ON THE INACTIVATION OF ACTH BY PLASMA

Incubation time in minutes	Assay animal								
	1			2			3		
	Milliunits of ACTH in standard, ACTH-plasma, or ACTH-plasma-L-cysteine mixtures injected								
	5 std.	5 10 ml. plasma	5 10 ml. plasma L-cyst	5 std.	5 10 ml. plasma	5 10 ml. plasma L-cyst	5 std.	5 10 ml. plasma	5 10 ml. plasma L-cyst
	17-OHCS response in adrenal blood, μg./10 minutes								
Before incubation	10* 130			14* 127			10* 97		
2– 3		183			136			95	
42– 47			194			113			89
200–202		21			14			34	
237–239			94			69			102
274–275	165			63			89		
% fall from original value	0	89	52	50	90	39	8	64	0

* Control (nothing injected).

jected) collections recorded in Table 2 and hence had no ACTH activity. L-Cysteine did not affect the activity of standard ACTH solutions in one experiment (#3).

The initial and final assays for each mixture in each experiment are presented in Table 2. In each instance it can readily be seen that the steroidogenic activity of the "ACTH plus plasma" declined more rapidly than did the "ACTH plus plasma plus cysteine." The apparent decline in ACTH activity of the cysteine containing system of experiment #2 was not real but was due to a decline in the responsivity of the dog preparation during the experimental day as is apparent in the decline in response to standard ACTH. Therefore, cysteine retarded or prevented the *in vitro* inactivation of hog ACTH by dog plasma.

This observation has been confirmed by an experiment in which heparinized blood was withdrawn from an adrenalectomized patient, T.M. (PBBH 3M357), 27 hours after the last steroid administration to ensure high levels of circulating ACTH. To one of two portions of the blood L-cysteine was added in a concentration of 10 gms. per 100 ml. Plasma from each portion was immediately chilled in ice. Ten ml. of each portion was assayed for ACTH content and the remainder incubated at 37° C for 215–220 minutes and then 10 ml. of each was reassayed in the same dog preparation. The plasma without L-cysteine gave an initial response of 109 μg. 17-OHCS/10 minutes, and after 220 minutes at 37° C, 37 μg. 17-OHCS/10 minutes. On the other hand, the plasma containing L-cysteine gave an initial response of 96 μg./10 minutes, and after 215 minutes at 37° C, 95 μg.

17-OHCS/10 minutes. Therefore, L-cysteine also prevented the inactivation of endogenous ACTH by human plasma.

DISCUSSION

The thermolability of the ACTH inactivating system and the temperature dependence of the rate of inactivation suggest that this activity is due to an enzymatic process. The observation on heat lability agrees with that of Pincus and co-workers (5) who destroyed an ACTH inactivating system in rat, human, and bovine blood by heating at 50° C for 30 minutes.

Whether enzymatic or not the inhibition of the inactivation by L-cysteine suggests that the process may be similar to the thiol reversible inactivation of ACTH by oxidation with hydrogen peroxide or ferricyanide as reported by Dedman, Farmer, and Morris (6). Since there are no detectable disulfide nor sulfhydryl groups in any known type of ACTH, the mechanism of thiol reversible oxidative inactivation of ACTH has to date remained an enigma (2). Kuehl (7) and co-workers showed that the potency of a corticotropin B fraction could be reduced from 100 units/mg. to 10–25 units/mg. by bubbling air through the solution overnight at a pH of 7.0 in the presence of ferric ions. In view of the fact that heating the plasma prior to the addition of ACTH prevented inactivation and the relatively short periods of incubation (4–6 hours) makes it unlikely that in the previous (1) and present report oxidation of ACTH by dissolved oxygen could have accounted for much of the inactivation.

ACTH inactivation could have been effected by hydrolytic cleavage of the molecule by a proteolytic enzyme or peptidase of serum with an L-cysteine sensitive redox center but this is not proven. White and Gross (8) showed that bovine fibrinolysin at a pH of 6.0–7.5 would split corticotropin A in two positions rendering it physiologically inactive (by ascorbic acid depletion). They also cite an observation of Pincus that the heat labile ACTH inactivating factor which he had previously reported (5) could be traced to plasma fraction III. Such observations have recently been strengthened by the report of Mirsky and colleagues (9) in which they point out that human plasma, activated by streptokinase, will degrade ACTH in a manner similar to streptokinase activated plasminogen or plasmin (fibrinolysin) alone. They did not report physiological activity changes in incubated media. They found that plasma alone, without added streptokinase, degraded less than 10% of the protein of the ACTH preparation at 37° C in two hours. Therefore, it is unlikely that the inactivation presently reported is of the same nature. Fekete (10, 11) has presented some evidence that protamine *in vitro* can complex with ACTH and inactivate its ascorbic acid depleting action. That such a mechanism (i.e., complexing of ACTH with another protein moiety of the blood masking physiological activity) is involved in the inactivation of ACTH by plasma remains a possibility.

Acknowledgments

We are indebted to Dr. George W. Thorn for his interest and advice during this investigation. The technical assistance of Mrs. N. Meegan, Miss N. Madeira, and Mrs. J. Kolar was very much appreciated.

REFERENCES

1. Meakin, J. W., W. H. Tingey, Jr. and D. H. Nelson: *Endocrinology* **66:** 59. 1960.
2. Behrens, O. K. and W. W. Bromer: *Ann. Rev. Biochem.* **27:** 57. 1958.
3. Nelson, D. H. and D. H. Hume: *Endocrinology* **57:** 184. 1955.
4. Nelson, D. H. and L. T. Samuels: *J. Clin. Endocrinol. and Metab.* **12:** 519. 1952.
5. Pincus, G., T. F. Hopkins and O. Hechter: *Arch. Biochem.* **37:** 408. 1952.
6. Dedman, M. L., T. H. Farmer and C. J. O. R. Morris: *Biochem. J.* **66:** 166. 1957.
7. Kuehl, F. A., M. A. P. Meisinger, N. G. Brink and K. Folkers: *J. Am. Chem. Soc.* **75:** 1955. 1953.
8. White, W. F. and A. M. Gross: *J. Am. Chem. Soc.* **79:** 1141. 1957.
9. Mirsky, I. A., G. Perisutti and N. C. Davis: *J. Clin. Investigation* **38:** 14. 1959.
10. Fekete, G.: *Experienta* **11:** 310. 1955.
11. Fekete, G.: *Experientia* **12:** 222. 1956.

ISOLATION AND IDENTIFICATION OF BILE ESTROGENS IN PREGNANT WOMEN

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ABSTRACT

1. In extracts of bile obtained by duodenal intubation from 17 Rh-sensitized apparently healthy pregnant women at full-term, estriol and estrone have been isolated and identified.
2. The bulk of estriol was found to be predominantly in a "conjugated" form.
3. Presumptive evidence has been obtained, which seems to indicate that 16-*epi*estriol and estradiol-17β are also present in such extracts.

ALTHOUGH estrone has been isolated from the bile of pregnant cows (1), attempts to isolate steroid estrogens from the bile of pregnant women have been unsuccessful so far. Thus Pearlman & Rakoff (2) did not succeed in isolating estrogens from more than 20 liters of bile collected from women in the last trimester of pregnancy, although extracts of such bile exhibited a strong estrogenic activity. Since in partition studies some degree of estrogenic activity was also found in the strongly acidic phenol fraction, these workers suggested that estriol might be present in such extracts.

We have now attempted to isolate and identify some of the estrogens in extracts of human pregnancy bile at full-term. The results reported in this paper indicate that in addition to large amounts of estriol, estrone and probably also estradiol-17β and 16-epiestriol are present in such extracts.

EXPERIMENTAL

Material.—Bile has been obtained at full-term from 17 Rh-sensitized pregnant women, who volunteered for the collection of bile. On routine clinical examination they were found to be apparently healthy. The bile was obtained in the morning before breakfast by duodenal intubation following the introduction of a few ml of ethyl ether. It was found to be rather difficult to obtain large amounts of bile in this way from the patients; the amount obtained varied between 5.3 and 92 ml., averaging 37 ml. Although efforts have been made to avoid admixture of duodenal secretions, the presence of some duodenal fluid in some of the samples cannot definitely be excluded.

Hydrolysis and extraction.—The collected bile was diluted with distilled water to 100–300 ml., and immediately hydrolyzed for 60 minutes with 15 vol% of HCl, the acid being added to the boiling aqueous solution through a reflux condenser. It was then chilled

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under tap water and extracted 4 times with freshly distilled ether, according to the specifications of Brown (3). The ether extracts were combined and processed according to Brown (3) and Brown *et al.* (4) with slight modifications described previously (5), including also a toluene–sodium hydroxide partition (6).

Chromatography of estrogen methyl ethers.—This has been carried out according to Brown (3) as modified by Diczfalusy & Westman (5).

Countercurrent distribution.—All K-values have been calculated from 24 transfer countercurrent distributions. An all-glass apparatus according to Craig & Post (7) has been used. Partition coefficients were calculated according to Williamson & Craig (8) and theoretical distribution values according to Way & Bennett (9).

Color reactions.—The modified Kober reaction was carried out according to Brown (3) with the exception that for reading of low optical densities 50 mm. long cells have been used as previously suggested (10). Extraction of the Kober color was carried out according to Ittrich (11), using the modification of Salokangas & Bulbrook (12). This modification differs from the original technique of Ittrich in two respects: a) tetrachlorethane is used instead of chloroform, b) the use of ethanol is omitted. The modified extraction seems to yield a more stable color.[2]

The modified David (13) reaction has been carried out according to Axelson & Diczfalusy (14). In this modification estriol, or estriol methyl ether is heated with 0.15 ml. of a sulphuric acid-ethanol reagent (2.5%/v/v/99.9% ethanol in concentrated sulphuric acid) at 85° for 15 minutes. After cooling, 0.8 ml. of distilled water and 0.1 ml. of 85%/w/v/phosphoric acid are added, shaken, and the mixture is re-heated in a boiling water bath for 5 minutes. After cooling, 2.2 ml. of a 40% (v/v) aqueous ethanol is added and the blue color is read within an hour.

Sulphuric acid chromogens.—Approximately 60 μg. amounts of steroids were incubated with 3.0 ml. of concentrated sulphuric acid at room temperature for 2 hours and read in a Beckman spectrophotometer, model DU.

RESULTS

Isolation and identification of bile estrogens

Estriol.—When the purified extracts were partitioned according to Brown between benzene-petroleum ether and 2% ethanol, a Kober reaction carried out on an aliquot of the aqueous phase indicated the presence of considerable quantities of Kober chromogens. When this substance was distributed in a 24 transfer countercurrent distribution between benzene and water (10) the partition coefficient obtained (0.18) agreed with that of an authentic reference standard (0.19). Following methylation according to Brown (3) the product behaved chromatographically as estriol methyl ether. The methylated and chromatographed substance was then submitted to another countercurrent distribution between 40% (v/v/methanol and carbon tetrachloride. The K-value obtained (0.79) agreed reasonably well with that of the reference standard (0.82−0.85). Following acetylation with acetyl chloride, the methylated and acetylated product exhibited the same partition coefficient (K = 1.0) as the diacetate of estriol methyl ether, when partitioned between 90% methanol and n-hexane.

[2] We are indebted to Dr. R. D. Bulbrook (London) for making available these unpublished data to us.

On another portion of the methylated and chromatographed estriol methyl ether fraction different color reactions have been carried out. The Kober reaction ad modum Brown (3) revealed a distinct absorption maximum at 516 mμ., as shown in Figure 1. When the pink color was extracted with p-nitrophenol containing tetrachlorethane, the expected shift in absorption maximum to 537 mμ was obtained (Fig. 1). Furthermore, ultraviolet absorption spectrophotometry indicated the characteristic absorption spectrum of phenols. Following incubation for 2 hours with conc. sulphuric acid, the sulphuric acid chromogen obtained was identical with that obtained following the incubation of an authentic estriol methyl ether reference standard (Fig. 2).

Another portion of the methylated and chromatographed extract was used for the David color reaction (13, 14). The absorption spectra of the David chromogens obtained with this extract and with authentic estriol methyl ether are shown in Figure 3.

Finally, another sample of the methylated and chromatographed estriol

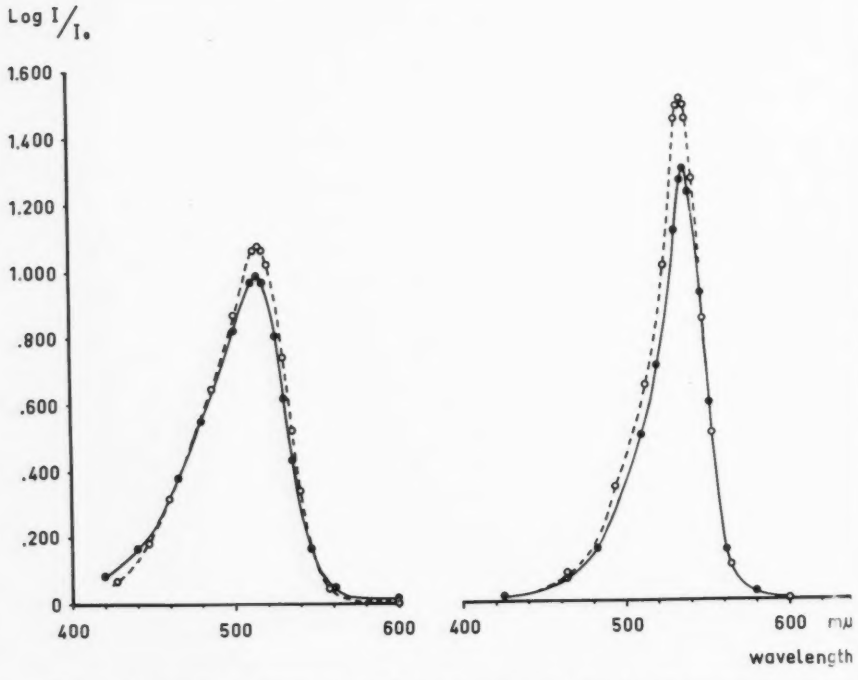

FIG. 1. Absorption spectrum of the Kober chromogen formed from an estriol methyl ether fraction isolated from human pregnancy bile (on the left) and the spectrum obtained following extraction of the pink color according to Ittrich (11), as modified by Salokangas & Bulbrook (12) (on the right).

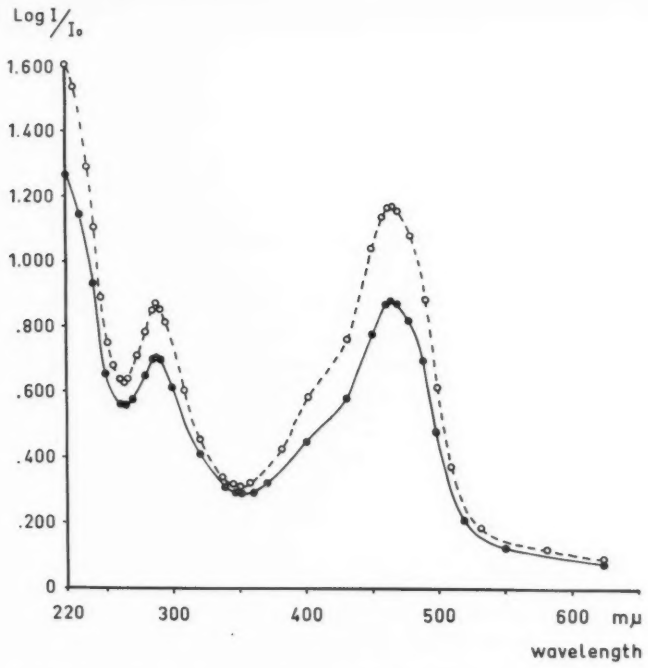

FIG. 2. Sulphuric acid chromogens obtained following incubation for 2 hours at room temperature of authentic estriol methyl ether (open circles) and of a methylated estriol fraction from pregnancy bile (filled circles).

methyl ether was submitted to infrared spectrometric identification.[3] The infrared spectrum agreed completely with that of authentic estriol methyl ether.

Thus the identification of estriol in extracts of human pregnancy bile is based on the following evidence:

1) Its behavior in the "Brown" partition
2) The partition coefficient of the "free" compound
3) The partition coefficient of its methyl ether
4) The partition coefficient of the acetylated methyl ether
5) The chromatographic behavior of the methylated compound
6) The absorption spectrum of the methylated compound following the Kober reaction according to Brown
7) The characteristic change in the absorption maximum of the pink color following extraction

[3] We are indebted to Dipl. Eng. S. Veige, AB Leo (Hälsingborg) for carrying out the infrared spectrometric identification.

8) The absorption spectrum of the methylated compound in the ultraviolet light
9) The absorption spectrum of the sulphuric acid chromogens
10) The absorption spectrum of the David chromogens
11) The infrared absorption spectrum.

In view of the accumulated evidence it is therefore concluded that the isolated compound was indeed identical with estriol.

Estrone.—Following the Brown partition the benzene-petroleum ether phase was evaporated and the residue methylated according to the tech-

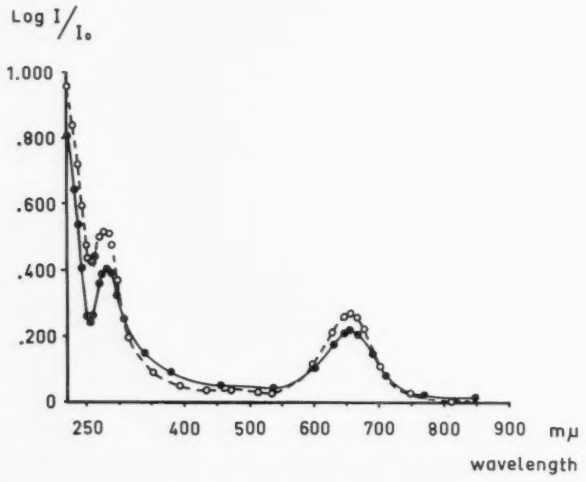

FIG. 3. Absorption spectrum of the David chromogens formed from authentic estriol methyl ether (open circles) and from a methylated estriol fraction of pregnancy bile (filled circles).

nique of Brown (3). When the methylated fraction was chromatographed on partially deactivated alumina, the presence of an estrone methyl ether-like Kober chromogen was indicated. A Kober reaction carried out according to Brown gave an absorption maximum at 516 mμ, which showed the characteristic shift from 516 mμ to 536 mμ following an Ittrich extraction (11, 12).

When a Girard separation was carried out, subsequent Kober reaction indicated that virtually all Kober chromogenicity was present in the ketonic fraction. Following reduction of this methylated ketone with KBH_4 in aqueous methanol, the absorption maximum of this Kober chromogen shifted to 518 mμ, which is characteristic for estradiol-17β. When the reduced compound was submitted to a countercurrent distribution in the following solvent system: n-hexane: 100, methanol: 90, water: 10, the K-

value obtained (0.22) agreed with that of authentic estradiol-17β 3-methyl ether (0.22). Following acetylation with acetyl chloride in benzene, the product partitioned itself in the same solvent system with a K-value of 2.5. An authentic sample of acetylated crystalline estradiol-17β 3-methyl ether distributed simultaneously in this system also gave a K-value of 2.5.[4]

Thus the view that the estrone methyl ether-like Kober chromogen present in extracts of human pregnancy bile is identical with estrone methyl ether is based on the following evidence:

1) Chromatographic behavior of the methylated compound
2) The absorption spectrum of the Kober chromogen
3) The absorption spectrum of the Kober chromogen following a modified Ittrich extraction
4) The behavior of the methylated compound in the Girard separation
5) The shift in the absorption maximum of the Kober chromogen from 516 to 518 mμ following reduction with potassium borohydride
6) The partition coefficient of the reduced estrone methyl ether
7) The partition coefficient of the reduced and acetylated estrone methyl ether.

Due to the small amount of material available (approximately 5μg./100 ml.) no infrared spectrometric identification could be carried out. Nevertheless, in view of the accumulated evidence it seems justifiable to conclude that the isolated substance was identical with estrone methyl ether.

Estradiol-17β.—Chromatography of the methylated estrone+estradiol-17β fraction indicated also the presence of small amounts of estradiol-17β. However, the amount of this compound was only approximately 10 per cent of that of estrone. The absorption spectrum of this Kober chromogen had its maximum at 518 mμ. which shifted to 538 mμ following a modified Ittrich extraction. Lack of material excluded the possibility of further characterization of this compound. (The concentration of estradiol-17β was less than 1 μg./100 ml.) Thus, in view of the high specificity of the Brown method, it is suggested that these data may be taken as presumptive evidence in favor of the view that estradiol-17β might also be present in extracts of human pregnancy bile. It is realized, however, that the evidence available does not allow of any definite conclusions.

16-Epiestriol.—When—following a Brown partition—the aqueous phase

[4] It should be noted that the constant of estradiol-17β 3-methyl ether (K=0.22) and that of estradiol-17β 3-methyl ether-17 acetate (K=2.5) in this system are lower than those reported previously (K=0.27 and 3.5 respectively) in a similar solvent system (5,6). The higher K-values obtained in the previously used solvent system (n-hexane /90% (v/v) methanol) are attributable to the contraction in volume of methanol following the addition of water, resulting in a relatively higher water content of the lower phase.

was extracted with ether, evaporated and partitioned between benzene and water, the countercurrent distribution indicated, in addition to huge amounts of estriol, also the presence of small amounts of another Kober chromogen exhibiting a K-value of 3.2 (Fig. 4). This value of partition coefficient is very close to that (K = 3.4) described by Diczfalusy & Halla (10) and agrees with that (K = 3.2) reported by Diczfalusy & v. Münstermann (15). The extremely small amount of material available (1 to 3 μg./100 ml. bile) excluded further characterization of this compound. Thus—although the evidence is not sufficient for a definite identification—it would appear

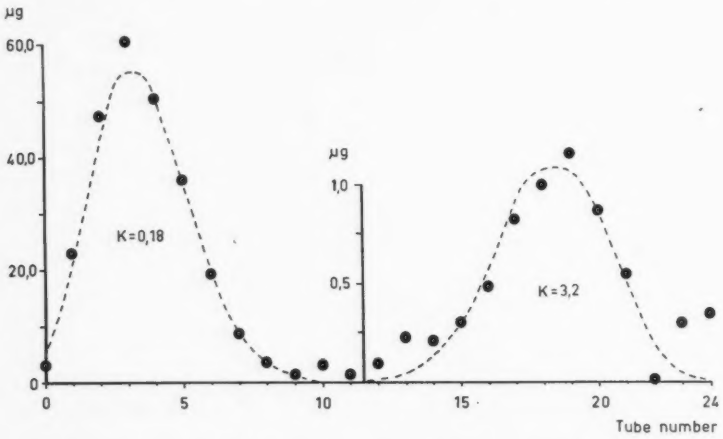

FIG. 4. Countercurrent distribution of the estriol- and 16-*epi*estriol-like Kober chromogens obtained from a pooled extract of human pregnancy bile. Solvent system: benzene (upper phase) and water (lower phase). K = partition coefficient. Dotted line indicates theoretical distribution values, calculated according to Way & Bennett (9). The readings on the right have been taken with 50 mm. long cells.

from these data that some 16-epiestriol might also be present in such bile extracts.

QUANTITATIVE INVESTIGATIONS

The purpose of the experiments described in this section was to obtain some preliminary information as to the nature and approximative amount of estrogen present in pregnancy bile. In order to carry out strictly quantitative assays a method would have been needed which fulfills the recognized criteria of reliability. Work is now in progress on the development of such a method (16).

Since it was found that the Brown methods (3, 4) are not suitable for the estimation of estrone and estradiol-17β in human bile, but that reliable estimates of estriol can be obtained if the toluene–N NaOH partition (6) is included in the Brown procedures, the present assays have been re-

stricted to the estimation of estriol. This was assayed in 17 pregnancy bile samples, obtained at full term from 17 volunteers. A very great individual variation was found, with estriol values ranging from 1.6 to 604 μg./100 ml. bile. Assuming a lognormal (17) distribution of individual estimates, the (geometric) mean concentration of estriol in pregnancy bile at term can be tentatively estimated as 72 μg./100 ml. However, since no simultaneous recovery experiments have been conducted on pregnancy bile, all recoveries being restricted to bile specimens from non-pregnant individuals, it should be pointed out that the above values may represent underestimates. Data obtained at the "quantitative characterization" (6) of such estriol fractions indicate, on the other hand, that these values can hardly represent gross overestimates.

On basis of the isolation studies carried out on different pools of pregnancy bile a tentative estimate of the approximate relationship between different biliary estrogens in pregnancy may also be calculated. Thus from the preliminary data available it would appear that there is at least 30 times as much estriol as estrone or 16-*epi*estriol in such extracts, whereas the concentration of estradiol-17β appears to be very low, probably less than 1.0 μg. per 100 ml. bile. Thus—as far as it can be judged from these preliminary data—it would appear that the estrogen pattern of human pregnancy bile differs from that of pregnancy urine in that less estrone+estradiol-17β in relation to estriol seem to be present in the bile. It is realized that further work is needed before this problem can be definitely clarified.

Attempts have also been made to separate "free" from "conjugated" estriol, using ether or chloroform extraction of diluted, unhydrolyzed bile prior to acid hydrolysis. No "free" estriol could be detected in 3 out of 4 such experiments, whereas in the fourth one the concentration of "free" estriol was 10 per cent of that of total estriol. Thus it would appear that the estriol of human pregnancy bile at full term is present predominantly in a "conjugated" form. The nature of conjugation has not been investigated in the present study. Work is, however, in progress on this problem.

DISCUSSION

Since 1943 when Cantarow *et al.* (18) reported for the first time that the estrogenic activity of liver bile obtained by duodenal intubation of women at full-term pregnancy is approximately three times that of the blood, it was generally assumed that human pregnancy bile must contain a fairly high amount of estrogen. The experiments of Pearlman & Rakoff (2) confirmed the data of Cantarow *et al.* (18) and indicated a high estrogenic activity of diluted pregnancy bile when assayed by the vaginal smear technique in spayed mice.

The data reported in this paper confirm and extend the findings of these investigators and demonstrate that estriol and estrone can be isolated from samples of human pregnancy bile. In addition, the present data indicate

that some other estrogens, such as estradiol-17β and 16-*epi*estriol, might also be present in such extracts.

All the patients employed in this investigation were apparently healthy Rh-sensitized mothers. That Rh-sensitized mothers should greatly differ from non-sensitized pregnant women as far as their estrogen metabolism is concerned, does not seem to be very likely. At any rate, the urinary excretion of estrone, estradiol-17β and estriol in such sensitized women does not show any major deviations from that observed in normally pregnant women (19). Therefore, it seems justifiable to assume that the bile of normally pregnant women also contains significant amounts of these estrogens.

As far as the quantitative aspects of the problem of estrogens in pregnancy bile are concerned, it is felt that it would be somewhat premature to draw any definite conclusions from the results reported in this paper. On the other hand, it seems justifiable to suggest that the bulk of estriol in pregnancy bile occurs in a "conjugated" form.

Acknowledgment

We are indebted to Dr. R. D. Bulbrook (London) for making some unpublished data available for us. Our thanks are also due to Dipl.Eng. S. Veige, AB Leo (Hälsingborg) for carrying out the infrared spectrometric identification. We would also like to thank Dr. E. Menini and Miss B. Martinsen for their co-operation in some of the experiments. One of us (H. A.) is indebted to the Nordisk Insulinfond and to the Finska Läkaresällskapet for personal grants.

REFERENCES

1. Pearlmann, W. H., A. E. Rakoff, A. Cantarow and K. E. Pashkis: *J. Biol. Chem.* **170:** 173. 1947.
2. Pearlmann, W. H. and A. E. Rakoff: *Endocrinology* **44:** 199. 1949.
3. Brown, J. B.: *Biochem. J.* **60:** 185. 1955.
4. Brown, J. B., R. D. Bulbrook and F. G. Greenwood: *J. Endocrinol.* (London) **16:** 49. 1957.
5. Diczfalusy, E. and A. Westman: *Acta endocrinol.* **21:** 321. 1956.
6. Diczfalusy, E. and P. Lindkvist: *Acta endocrinol.* **22:** 203. 1956.
7. Craig, L. C. and O. Post: *Anal. Chem.* **21:** 500. 1949.
8. Williamson, B. and L. C. Craig: *J. Biol. Chem.* **168:** 687. 1947.
9. Way, E. L. and B. M. Bennett: *J. Biol. Chem.* **192:** 335. 1951.
10. Diczfalusy, E. and M. Halla: *Acta endocrinol.* **27:** 303. 1958.
11. Ittrich, G.: *Z. Physiol. Chem.* **312:** 1. 1958.
12. Salokangas, R. A. A. and R. D. Bulbrook: personal communication.
13. David, K.: *Acta brev. Neerl.* **4:** 64. 1934.
14. Axelson, E. and E. Diczfalusy: to be published.
15. Diczfalusy, E. and A.-M. v. Münstermann: *Acta endocrinol.* in press.
16. Adlercreutz, H., Jr.: in preparation.
17. Gaddum, J. H.: *Nature* **156:** 463. 1945.
18. Cantarow, A., A. E. Rakoff, K. E. Paschkis, L. P. Hansen and A. A. Walking: *Proc. Soc. Exp. Biol. & Med.* **52:** 256. 1943.
19. Diczfalusy, E., Menini and L. Engström: to be published.

THE EFFECTS OF UNILATERAL OVARIECTOMY ON FOLLICULAR MATURATION IN THE HAMSTER

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ABSTRACT

In the hamster, the follicles that will ovulate at any one estrus can usually be distinguished by the third day of the estrous cycle. This has been shown by determining whether compensatory ovulation occurs after unilateral ovariectomy on the various days of the estrous cycle. The results indicate that unilateral ovariectomy on the first and second day of the cycle is followed by a doubling in the number of ova released from the remaining ovary. There is no compensatory increase in ovulation after unilateral ovariectomy on the third or fourth day. However, if pregnant mare serum or human chorionic gonadotropin is injected at the time of unilateral ovariectomy on the third day the ovulation rate is doubled in the remaining ovary. The altered responsiveness of the ovary in the latter half of the cycle is paralleled by accelerated growth of the largest follicles as the antra begin to expand.

It is believed that a shift in the ratio of FSH and LH at midcycle accounts for the difference in results when unilateral ovariectomy is performed during the first or second half of the estrous cycle.

WHEN, during the estrous cycle, can the follicles that will ovulate at the next estrus be identified? Previous attempts to answer this question indirectly have been based on the growth of the largest vesicular follicles during the estrous cycle (1–3). The present investigation approaches the problem in another way by determining the effects of unilateral ovariectomy on the number of ovulations from the surviving ovary.

It has long been known that unilateral ovariectomy (semispaying) leads to compensatory hypertrophy of the remaining ovary and that the ovulation rate characteristic of the species remains remarkably constant after this procedure (4–8). In previous investigations, several weeks elapsed between unilateral ovariectomy and the time that the remaining ovary was finally removed. In contrast, in the experiments to be reported here, unilateral ovariectomy was carried out for each day of the estrous cycle and the remaining ovary and oviduct were removed shortly after the next expected ovulation.

MATERIALS AND METHODS

The Effects of Unilateral Ovariectomy on Ovulation

Twenty-eight sexually mature golden hamsters (*Cricetus auratus*) were used to establish the effects of unilateral ovariectomy on ovulation from the remaining ovary. The

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duration of the estrous cycle was determined by examining the vaginal smears; the voluminous thick discharge at the approximate time of ovulation was especially valuable in dating the cycle (9). Only those animals which had completed at least two consecutive 4-day cycles were used in the experiments. In this paper, day 1 designates the morning of ovulation while day 4 is the morning before the next expected ovulation. In the hamster, ovulation usually takes place between 1 and 4 A.M. (10–12).

The hamsters were divided into four equal groups and the *right ovary* was removed by the dorsal route at either day 1, 2, 3, or 4, between 9 and 10 A.M. The animals were killed the day after the next expected ovulation between 9 and 10 A.M., and the *left oviduct* was then removed. Serial sections cut at 10 μ and stained with Heidenhain's azan were prepared of all of the ovaries and oviducts. The number of corpora lutea in the right ovary was then compared with the number of ova in the corresponding left oviduct.

The Growth of the Vesicular Follicles

The next step was to establish the rate of growth of the largest follicles to determine whether their morphological development could be correlated in any way with the response to unilateral ovariectomy.

Twenty-six serially sectioned ovaries were studied to establish the pattern of follicular growth. The mean volume of the six largest follicles in each ovary was calculated by measuring the two diameters at right angles to each other in the largest section of each follicle. A third diameter was determined by counting the number of serial sections comprising the follicle. The average of the three values was then substituted in the formula for a sphere: $1/6\pi D^3$.

The Effects of PMS and HCG on Ovulation

The third set of experiments were designed to determine whether exogenous gonadotropins would influence the effects of unilateral ovariectomy. Immediately after semispaying on day 3, a series of 22 hamsters were injected subcutaneously with varying dosages of pregnant mares' serum (PMS) or human chorionic gonadotropin (HCG)[2] (see Table 2 for details). The animals were killed on day 2 of the next cycle and comparisons were again made between the number of corpora lutea in the previously removed right ovary and the number of ova in the left oviduct.

RESULTS

The effects of unilateral ovariectomy on ovulation

When unilateral ovariectomy was performed during the first two days of the estrous cycle, twice as many ova were ovulated from the left ovary than from the previously removed right ovary (Table 1). In contrast, after semispaying on the third or fourth day of the cycle, there was no difference in the number of ova ovulated between right and left ovaries. The difference in the number of ovulations was highly significant when the left ovaries were compared for the first and second halves of the estrous cycle (for the left ovaries of day 1 and 3, $t = 4.3$; $P < .001$). However, there was no significant statistical difference between any of the means of the right ovaries.

Unilateral ovariectomy did not change the interval between ovulations.

[2] The PMS and HCG (APL^{Rx}) were generously furnished by the Ayerst Laboratories, New York City.

TABLE 1. THE EFFECT OF UNILATERAL OVARIECTOMY ON THE NEXT OVULATION

Day of estrous cycle	No. of animals	No. of ova ovulated from *right ovary* (mean ± S.E.)	No. of ova ovulated from *left ovary* at next cycle (mean ± S.E.)
1	7	5.3 ± 0.30	11.1 ± 0.46
2	7	5.6 ± 0.38	10.9 ± 0.62
3	7	5.7 ± 0.93	6.7 ± 0.91
4	7	6.1 ± 0.66	6.6 ± 1.0

The hamsters invariably ovulated at the time predicted on the basis of their previous estrous cycles. One animal was unilaterally ovariectomized on day 1 and the next four cycles were exactly 4 days in length.

Growth of the ovarian follicles during the estrous cycle

At day 1, the mean volume of the largest follicles in the normal ovary was $12.2 \times 10^6\ \mu^3$ (Table 2). The granulosa cells were multilayered and there was no trace of an antrum; the theca was undeveloped and relatively avascular (Fig. 1). At day 2, the average follicular volume increased to $26.4 \times 10^6\ \mu^3$ and the antrum began to form as a series of separate cavities (Fig. 2). The most striking difference between the two groups of ovaries was the sudden increase in vascularity of the theca on the second day of the cycle. The vascularity of the theca was not limited to the larger follicles; many of the smaller follicles were also hyperemic. As shown in Table 2, there was a shift in the growth rate of the follicles between days 2 and 3 of the cycle. By day 3 the volume of the largest follicles had increased to $53.2 \times 10^6\ \mu^3$, due primarily to the formation of a large antrum (Fig. 3). The theca was even more intensely vascularized than at day 2. By day 4 the volume of the preovulatory follicles was $77.8 \times 10^6 \mu^3$ (Fig. 4). In summary, the increase in the growth rate of the follicles between days 2 and 3 was the result of the rapid expansion of their antra. The accelerated follicular growth coincided with the altered responsiveness of the remaining ovary to unilateral ovariectomy.

Why the remaining ovary did not undergo compensatory ovulation after unilateral ovariectomy in the second half of the cycle poses an interesting problem. Perhaps the largest follicles after antrum formation may somehow "inhibit" the growth of the remaining follicles and these vesicular folli-

TABLE 2. THE GROWTH RATE OF FOLLICLES IN THE HAMSTER OVARY

Day of estrous cycle	No. of animals	Average volume of largest follicles ($\times 10^6\ \mu^3$)	Range in volume of follicles ($\times 10^6\ \mu^3$)
1	6	12.2 ± 0.81*	8.8 to 21.3
2	6	26.4 ± 1.4	17.0 to 39.3
3	7	53.2 ± 3.7	41.0 to 78.5
4	7	77.8 ± 2.8	61.5 to 117.0

* Mean ± standard error of the mean.

PLATE 1

FIG. 1. Largest follicle on day 1 of the œstrous cycle of the hamster.
FIG. 2. Initial phase of antrum formation in day 2 follicle. Note the increased vascularity of the theca.
FIG. 3. Follicle from a day 3 ovary.
FIG. 4. Largest follicle from a day 4 ovary.
All photomicrographs are magnified 40 ×.

cles are the only ones capable of ovulation. However, after unilateral ovariectomy at day 3, there were many more medium sized follicles in the remaining day 4 ovary than in the corresponding normal ovary. This suggests that the surviving ovary was able to respond partially after semispaying in the latter half of the cycle, but not sufficiently to result in twice the number of ovulations.

An alternative possibility is that there may be a shift in the ratio of circulating FSH and LH at day 3 with a resultant predominance of LH. This agrees with current ideas on the gonadotropic control of ovulation (13). The following experiments were designed to test this possibility by determining whether PMS or HCG injected on day 3 could increase the number of ovulations.

The effect of PMS or HCG on ovulation after unilateral ovariectomy

Hamsters injected immediately after semispaying on day 3 with a single 15 I.U. dose of PMS, ovulated twice as many ova from the surviving left ovary as the non-treated group (Table 3). After the reduction of the PMS dosage from 15 to 5 I.U., the ovulation rate was still doubled. The number of ovulations after PMS treatment was in the same range as the number ovulated from the left ovary when unilateral ovariectomy was performed during the first half of the cycle (cf. Tables 1 and 3). The length of the cycle was unaffected by the administration of PMS; ovulation occurred in all instances at the expected time. When 15 I.U. of PMS was injected on day 3 into three *intact* hamsters, the animals averaged 14.0 ovulations for both ovaries (range: 11 to 16 ova). Cycle length was again unaffected by the PMS treatment.

When 12.5 I.U. of HCG was injected immediately after unilateral ovariectomy on day 3, the remaining ovary released even more ova than after the PMS treatments (Table 3). However, the estrous cycle was prolonged by one day among four of the HCG treated hamsters. Evidence of a delay

TABLE 3. THE EFFECT OF GONADOTROPINS ON THE NEXT OVULATION*

Treatment	Dosage (I.U.)	No. of animals	No. of ova ovulated from *right ovary* (mean ± S.E.)	No. of ova ovulated at next cycle from *left ovary* (mean ± S.E.)
None	—	7	5.7 ± .93	6.7 ± .91
PMS	15.0	6	6.0 ± .58	11.7 ± .60
	5.0	5	5.4 ± .60	10.2 ± 1.1
HCG	12.5	6	5.8 ± .96	15.0 ± 1.9†
	5.0	5	6.0 ± .44	9.0 ± 1.4

* After unilateral ovariectomy at day 3.
† Only 4 animals ovulated.

FIG. 4. Largest follicle from a day 4 ovary.
All photomicrographs are magnified 40 ×.

in ovulation was furnished by a 24 hour shift in the time of the post ovulatory vaginal discharge. In HCG treated hamsters killed on the day of the discharge the tubal ova were still closely surrounded by granulosa cells which again indicated that ovulation had only recently taken place. Two of the animals treated with 12.5 I.U. of HCG failed to ovulate. The ovaries at 24 hours after the expected ovulation contained large vacuolated corpora lutea. The short normal life span of the corpora lutea of ovulation had apparently been prolonged by the HCG treatment.

When the HCG dosage was reduced from 12.5 to 5 I.U., there was no significant difference between the ovulation rates of the right and left ovaries. Of the five animals tested at this level, three showed a normal duration of the estrous cycle; in all three cases the number of ovulations did not differ between right and left ovaries. In the remaining animals in which the cycle *was* prolonged by one day, the surviving left ovary ovulated at a higher rate than the right one (for female 84, right ovary: 7 ova, left ovary: 11; for female 86, right ovary: 6 ova, left ovary: 13).

DISCUSSION

The present experiments indicate that in the hamster the follicles that will ovulate during a specific estrous cycle are usually set aside by the third day. After unilateral ovariectomy on days 1 or 2, the remaining ovary doubled its normal ovulation rate, but there was no increase in ovulation when semispaying was carried out on days 3 or 4 (Table 1).

Simultaneous with a change in the responsiveness of the ovary in the latter half of the cycle there was an increase in the growth rate of the largest vesicular follicles. The accelerated follicular growth resulted from the rapid expansion of the antrum between days 2 and 3. In the mouse, an increase in the growth of the largest follicles also occurs two days before ovulation (1) and it has been suggested that these follicles will ovulate at the next estrus. Similarly, in the rat, follicular volume doubles between 48 and 72 hours after the beginning of heat (3).

The hamster was the ideal animal for a study of this type in that ovulation occurs at a remarkably constant 4-day interval. The duration of the estrous cycle is not altered after unilateral ovariectomy and the rapid regression of the corpora lutea of ovulation means that there is never more than one generation of large corpora lutea in the ovary. Preliminary experiments with the white rat have so far been complicated by the inability to consistently fulfill these requirements.

In the present short term experiments, the effects of unilateral ovariectomy on the follicular population have not been established. Several months after prepubertal rats are semispayed, the surviving ovary is more than 100% heavier than control ovaries (after the appearance of corpora lutea) (4). Furthermore, there is no difference in the total number of ova in surviving or control ovaries. The important distinction is that the number

f well-developed follicles is greater in semispayed than in control ovaries.

An increase in the population of large follicles after unilateral ovariectomy may be caused by (1) a decrease in follicular atresia so that more follicles may reach maturity; (2) an increased proliferation of medium sized follicles or by (3) a combination of factors 1 and 2. In the rabbit, decreased follicular atresia is mainly responsible for compensatory ovulation (5). A quantitative study of the proportion of follicles in different stages of development is necessary before the relative importance of these factors can be determined for the hamster.

The surviving ovary, after unilateral ovariectomy at day 3, ovulated twice as many ova as the control ovary following the injection of either PMS or HCG (Table 3). While it is thought that PMS is predominantly an FSH preparation and HCG is mainly effective as an LH compound (14), in these experiments both gonadotropins were able to increase the ovulation rate.

It is possible that the hormones may be increasing ovulation by different mechanisms. With PMS administration, perhaps more follicles are initially stimulated to grow, but the length of the estrous cycle is unaltered. With HCG treatment, the prolongation of the cycle suggests that more follicles may have time to reach maturity. The HCG treatment may result in an increase in the *final* number of ripe follicles rather than a greater initial proliferation of growing follicles. It is suggested that a possible luteotropic effect of HCG in the hamster (as evidenced by the longer histological survival of the corpora lutea of ovulation) could account for the lengthening of the estrous cycle. Further quantitative study of the ovary is needed to determine whether this distinction between the effects of PMS and HCG is valid.

REFERENCES

1. Brambell, F. W. R.: *Proc. Roy. Soc., London ser. B*, **103**: 258. 1928.
2. Dempsey, E. W.: *Am. J. Physiol.* **120**: 126. 1937.
3. Boling, J. L., R. Blandau, A. Soderwall and W. Young: *Anat. Rec.* **79**: 313. 1941.
4. Arai, H.: *Am. J. Anat.* **28**: 59. 1920.
5. Asdell, S. A.: *Brit. J. Exp. Biol.* **1**: 473. 1924.
6. Lipshutz, A.: *Biological Rev.*, **2**: 263. 1927.
7. Slonaker, J. R.: *Am. J. Physiol.* **81**: 620. 1927.
8. Desaive, P.: *Archiv. de Biol.* **60**: 137. 1949.
9. Deansely, R.: *Proc. Zool. Soc., London ser. A*, **108**: 31. 1938.
10. Ward, M. C.: *Anat. Rec.* **94**: 139. 1946.
11. Austin, C. R.: *J. Roy. Microscop. Soc.* **75**: 141. 1956.
12. Strauss, F.: *J. Embryol. Exp. Morph.* **4**: 42. 1956.
13. Hisaw, F. L.: *Physiol. Rev.* **27**: 95. 1947.
14. Hamburger, C.: *Acta Endocrinol.* **31**: 59. 1957.

PITUITARY LACTOGENIC HORMONE CONCENTRATION DURING PREGNANCY IN THE RAT[1]

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ABSTRACT

A study was made in rats to ascertain whether the growth and secretory phases of the mammary gland during pregnancy were associated with variation in concentration of pituitary lactogenic hormone. Pituitary lactogenic hormone levels were determined by the common pigeon micro method of assay. Pituitary weight was low during most of pregnancy, but increased markedly a short time before parturition (day 21). Pituitary lactogenic hormone increased during the first two-thirds of pregnancy during which time there was growth, but little secretory activity of the mammary gland. Peak concentration (.051 I.U. /mg. pituitary) was reached on day 16. As the secretory activity of the mammary gland progressively increased during the last one-third of pregnancy, pituitary lactogenic hormone levels decreased markedly reaching the lowest point (.004 I.U./mg.) on day 21. The significance of this is discussed. The influence of 1, 3.6, 15 and 50 μg./day estradiol benzoate (E.B.) for 5 days upon pituitary weight and lactogenic hormone concentration was studied in mature female rats. Greatest pituitary enlargement occurred with 15 μg./day E.B. while pituitary lactogenic hormone increased in concentration as the log-dose of E.B. The level resulting from 1 μg./day E.B. (.054 I.U./mg.) closely approximated the highest level found during pregnancy.

THERE is a tendency to think of milk secretion as primarily occurring only after parturition. However, biochemical and cytological evidence indicate that during the latter third of pregnancy an increase in secretory activity occurs (1–4). It has been shown, for example, that mammary gland alveoli of rats become progressively distended with secretory products during late pregnancy with maximum engorgement occurring a few hours before parturition (3). Since initiation of milk secretion has been shown to be dependent upon lactogenic hormone released from the adenohypophysis (5, 6), it seemed of interest to investigate whether prepartum secretory activity of the mammary gland would be reflected in an alteration in pituitary concentration of lactogenic hormone from that found during the first two-thirds of pregnancy.

MATERIALS AND METHODS

Adult primiparous female rats of the Sprague-Dawley-Rolfsmeyer strain were housed under conditions of unifrom temperature (78 ± 1° F) and fed Purina Lab Chow and water *ad libitum*.

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Experiment I.—Female rats weighing 250–260 gm. were bred with males of the same strain. Ten rats were killed on each of days 12, 16 and 18 of pregnancy. Ten others were killed a few hours before parturition (approximately day 21). Immediately after sacrifice, each pituitary gland was removed, blotted to remove surface moisure, weighed to the nearest .1 mg. on a Roller-Smith torsion balance, and frozen until assayed for lactogenic hormone. Pituitary glands similarly obtained from non-pregnant female rats of the same age and weight range served as controls.

Experiment II.—Sixty female rats weighing 250–265 gm. were injected subcutaneously each day for 5 days with 1.0, 3.6, 15.0 or 50.0 μg. estradiol benzoate (E.B.) in .1 ml. olive oil. Fifteen served as controls and were injected with .1 ml. olive oil/day for a similar period. Twenty-four hours after the last injection each rat was killed and its pituitary gland removed and handled as described above. Each pregnant and E.B.-treated animal was skinned and its mammary glands examined for extent of development and/or secretory activity.

Assay of Lactogenic Hormone—Five pituitary glands were pooled for each assay. These were allowed to thaw at room temperature, crushed in an agate mortar and suspended in distilled water. The suspension was then injected intradermally in .1 ml. volume each day for 4 days over one of the crop-sacs of 15 adult common pigeons of both sexes weighing 270–320 gm.

Distilled water solutions of standard lactogenic hormone[3] containing 20 I.U./mg. were similarly injected in doses of .18, .7, 2.8 or 5.6 μg./pigeon/day over the opposite crop-sacs in some of the assays.

Twenty-four hours after the last injection each pigeon was killed, their crop-sacs removed and cut into halves. Each half then was stretched fully over a light source and the diameter of proliferation measured. Crop-sac responses resulting from injection of the pituitary suspension were converted to mg. lactogenic hormone by the regression equation $Y = 1.088 + .842 (\log 10^{-4}X)$. This was calculated on the basis of the diameter of proliferation (Y) of the crop-sacs of pigeons which were injected with various levels of standard lactogenic hormone (X). Conversion to I.U. was accomplished by multiplying the value of X by 20 (20 I.U./mg.).

RESULTS

Weight of pituitary glands and their concentration of lactogenic hormone of non-pregnant and pregnant rats are shown in Figure 1. Average pituitary weight of rats pregnant 12, 16, or 18 days was less than that of non-pregnant controls. The difference was significant ($P = .01$) in each instance. On day 21, average weight of pituitary gland was significantly greater ($P = .001$) than those of rats 12, 16, or 18-days pregnant, but did not differ significantly from those of non-pregnant or 2-day lactating rats. Pituitary lactogen concentration on day 12 of pregnancy averaged .014 I.U./mg. pituitary or only 34% of that present in pituitary glands of non-pregnant female rats. The peak concentration of lactogenic hormone was reached on day 16 of pregnancy (.051 I.U./mg.). Beyond this day the level was reduced to the point where only 8% of that present on day 16 found in pituitary glands of rats on day 21 of pregnancy though, at this time, pituitary weight was maximum.

In a second experiment injection of estradiol benzoate (E.B.) into ma-

[3] The lactogenic hormone (NIH-SP-1) is a highly purified sheep preparation being distributed by the National Institutes of Health.

ture female rats in dose of 1, 3.6, 15 or 50 μg./day for 5 days caused significant increases in pituitary weight in comparison with controls (P = .001). Greatest pituitary enlargement occurred in those injected with 15 μg./day E.B. Lactogenic hormone concentration of pituitary glands of E.B.-treated rats varied directly with the log-dose of E.B. (Fig. 2). Pituitary glands of those injected with the greatest dose contained 102.6% more lactogenic hormone/mg. than those from controls. The lactogenic hormone concentration of .054 I.U./mg. obtained from pituitary glands of rats treated with 1 μg./day E.B. was approximately equal to the peak level of .051 I.U./mg. obtained on day 16 of pregnancy.

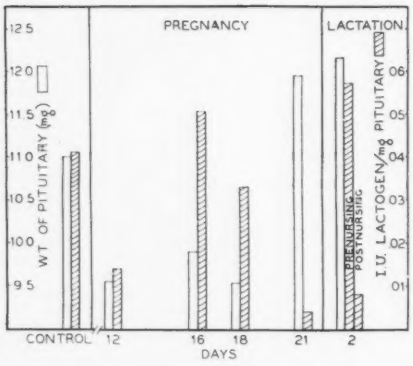

FIG. 1. Weight and lactogenic hormone concentration of pituitary glands of pregnant rats. Each point represents average of 10 glands. Data on day 2 of lactation from Grosvenor and Turner (14).

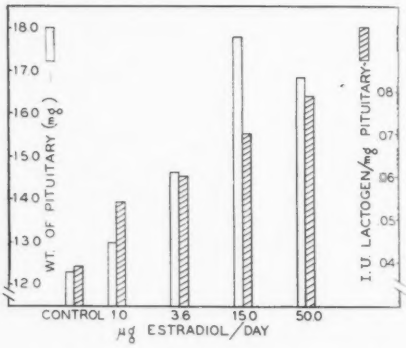

FIG. 2. Effect of estradiol (estradiol benzoate) upon pituitary weight and lactogenic hormone concentration of mature female rats. Estradiol benzoate injected daily for 5 days. Each point represents average of 15 glands.

Examination of mammary glands of 12 and 16-day pregnant rats showed good lobule-alveolar growth. The alveoli, however, were not distended to any extent. Glands from 18-day pregnant rats were slightly distended while those obtained from rats killed a few hours before parturition were very white in appearance and greatly distended with secretion. When the mammary glands were cut, the contained secretion exuded freely.

No alveoli were present or any secretion noted in the mammary glands of nonpregnant female rats injected with any level of E.B.

DISCUSSION

Previous studies of the pituitary lactogenic hormone content of the rat have shown that it is low during pregnancy but increases markedly after parturition (7, 8). Assay techniques employed in these studies were rather subjective, however, with the result that changes small in magnitude could not easily be detected. More sensitive assay methods for detection of lactogenic hormone have since become available (9, 10). With the use of one of

these (9) in the present study it has been shown that during the first two-thirds of pregnancy, during which time there was noted little or no evidence of secretory activity by the mammary gland, lactogenic hormone content of the pituitary gradually increased and reached a peak by day 16. In association with the observed progressive increase in secretory activity of the mammary gland during the last third of pregnancy there was a rapid decline in pituitary lactogenic hormone concentration of the pituitary.

These data suggest that a progressive accumulation of lactogenic hormone occurs during the period of mammary gland development. The hormone then is discharged during the latter part of pregnancy to initiate secretory activity of the mammary gland alveoli.

It is well known that estrogen secreted during pregnancy is necessary to insure mammary gland duct growth and, in synergism with progesterone, to promote full lobule-alveolar development. Reece and Turner (11) were the first to show that estrogens also could elicit an increase in lactogenic hormone content of the rat pituitary gland. This was later confirmed and extended to include other species (12–13). It would appear from the results of the present study that 1.0–3.6 μg./day estradiol benzoate is necessary to evoke a rise in pituitary lactogenic hormone comparable to the peak level of .051 I.U./mg. pituitary found on day 16 of pregnancy. It is of interest to note that these levels of estradiol benzoate approximate those generally employed in experimental development of rat mammary gland.

The factors which cause the prepartum release of lactogenic hormone from the pituitary are not known at the present time. It is known that following parturition the nursing stimulus elicits reflex release of the lactogenic hormone (14) and it is possible that some neural stimulus, possibly arising from the pregnant uterus, is instrumental in causing similar discharge during late pregnancy (Fig. 1). This possibility is presently being explored.

REFERENCES

1. Popjack, G. and M. L. Beeckman: *Biochem. J.* **46:** 547. 1950.
2. Folley, S. J. and A. L. Greenbaum: *Biochem. J.* **41:** 261. 1947.
3. Jeffers, K. R.: *Am. J. Anat.* **56:** 257. 1935.
4. Hammond, J.: The Physiology of Reproduction in the Cow. Cambridge University Press, Cambridge, 1927.
5. Meites, J. and C. W. Turner: *Am. J. Physiol.* **150:** 394. 1947.
6. Bradley, T. R. and P. M. Clarke: *J. Endocrinol.* **14:** 28. 1956.
7. Reece, R. P., I. L. Hathaway and H. P. Davis: *J. Dairy Sci.* **22:** 1. 1939.
8. Reece, R. P. and C. W. Turner: *Mo. Agr. Exp. Sta. Res. Bull.* 266. 1937.
9. Grosvenor, C. E. and C. W. Turner: *Endocrinology* **63:** 530. 1958.
10. Tanabe, Y., Y. Syoda and Y. Saeki: *Bull. of National Institute of Agr. Sci. Series G.* **9:** 57. 1954.
11. Reece, R. P. and C. W. Turner: *Proc. Soc. Exper. Biol. and Med.* **34:** 402. 1936.
12. Meites, J. and C. W. Turner: *Mo. Agr. Exp. Sta. Res. Bull.* **415.** 1948.
13. Meites, J. and C. W. Turner: *Mo. Agr. Exp. Sta. Res. Bull.* **416.** 1948.
14. Grosvenor, C. E. and C. W. Turner: *Endocrinology* **63:** 535. 1958.

SKELETAL METABOLISM IN ESTROGEN-TREATED RATS STUDIED BY MEANS OF Ca^{45} [1]

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ABSTRACT

Estradiol n-valerate, 0.5 mg. in oil, was given weekly to 50 immature rats and the effect upon the length of the spongiosa in the tibias and femurs was observed by x-ray. After 4 weeks 30 μc. of high specific activity Ca^{45} was given to the estrogen-treated rats and to an equal number of controls, which had received weekly injections of oil alone. Groups of 10 rats each were killed at intervals of from 1 hour to 8 days.

The distribution of Ca^{45} in the femurs, tibias and incisors was analyzed kinetically according to the method of Bauer, Carlsson, and Lindquist. In addition qualitative observations were made by microradiography and autoradiography of sections of the femurs.

The kinetic study, in agreement with the observations made by autoradiography, indicated a reduction of approximately 50% in the rate of resorption in the upper ends of the tibias of the estrogen-treated animals. This led to the conclusion that while removal of Ca from the central part of the spongiosa of the long bones was markedly inhibited, resorption was still progressing in the bones studied. Such resorption was presumably in locations in the bones in which growth and remodeling were prominent. It thus seems that resorption incident to remodeling is not appreciably affected by treatment with estrogens.

ADMINISTRATION of estrogens to young, growing rats produces an effect not observed in any other species. Resorption of the spongiosa in the long bones, which ordinarily serves to maintain the secondary spongiosa at an approximately constant length during growth, is inhibited. The result is that an elongated core of spongy bone is found in the metaphysis, its length depending upon the dose of estrogen, the period over which it is administered, and the rate of growth of the animal (1).

By use of tracer techniques, Bauer, Carlsson, and Lindquist (2) have been able to account for the radioactive calcium—Ca^{45}—in the skeleton of the animal to which it is administered, as the sum of the amount in the exchangeable fraction of the bone salt, plus the amount incorporated into the non-exchangeable fraction by accretion, less the amount removed by resorption.

The aims of the present investigation were: (a) to study the kinetics of

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the metabolism of calcium in growing rats after one of these physiologic variables, i.e., resorption, was interfered with by administration of a highly active estrogen preparation; and (b) to correlate the data so obtained with the information derived from autoradiography and microradiography.

MATERIALS AND METHODS

Eleven litters, each of 10 rats, of the Sprague-Dawley strain, thirty-three days old and of both sexes were used; they were fed a stock diet and each rat was housed in an individual cage. One litter was killed at the beginning of the experiment and provided untreated controls for the experimental animals.

Each of 10 litters was divided into 2 sub-groups. Five rats of each litter were each given 0.5 mg. of estradiol n-valerate (Delestrogen[4]) in 0.05 ml. of sesame oil intramuscularly once a week for 4 weeks. The remaining 5 rats of each litter were each given 0.05 ml. of sesame oil in the same manner. All rats were weighed twice a week. Roentgenograms of each rat were made at the time of each injection (Picker X-ray, portable type; No Screen Kodak Type AA film). At approximately 4 weeks after the initial dose of estrogen, all animals were given Ca^{45} by intraperitoneal injection. Each rat received 1.0 ml. of an isotonic saline solution containing about 30 μc. of high specific activity Ca^{45} (Oak Ridge, 3108 mc./gm.). One litter was killed at each of the following intervals: 1, 2, 4, 8, and 16 hours, and 1, 2, 4, 6, and 8 days.

Except for minor modifications described below, the techniques for the collection of samples and the determination of radioactivity were those of Carlsson (3). Under ether anesthesia the carotid artery was opened and blood was collected in a centrifuge tube until death of the animal. Total serum calcium was determined on individual blood samples from each rat by Clark and Collip's modification of the method of Tisdall (4). After titration the solution was brought to a volume of 3 ml. with dilute nitric acid (sp. gr. 1.084) and radioactivity was determined on a 2 ml. aliquot.

Femurs, tibias and incisor teeth were dissected out and the lengths of the bones were measured with a caliper. Roentgenograms were made of all specimens. Under x-ray control, the upper and lower ends of both tibias and fibulas were cut so as to include the elongated metaphyses within the end portions leaving the shaft free of medullary bone. The tibia-fibula segments are herein referred to as tibias. The intact right femurs, tibia segments and incisors were ashed in an electrically heated muffle and then weighed. The radioactive samples were dissolved in 5 ml. of dilute nitric acid and radioactivity was determined on a 3 ml. aliquot.

The aliquot samples were counted, using end-window G-M tubes (TGC 2, window thickness 1.6 and 1.8 mg./cm.^{2}). The radioactivity of the samples was compared with that of a standard solution of the isotope and the values were expressed as a percentage of the dose administered.

Sections of the left femurs of an estrogen-treated and an oil-treated rat from a 1-hour and from an 8-day interval (4 in all) were prepared for microradiograms and low resolution autoradiograms. The femurs were fixed in absolute alcohol, dehydrated with ether, embedded in methyl methacrylate and cut undecalcified with a high-speed rotating saw into sagittal sections of 100 microns. The sections were placed on Eastman Kodak Autoradiographic Type A plate and exposed for 3 days. The microradiograms were made by pressing the bone sections into contact with Eastman Kodak Spectroscopic Plate 649-0, exposed to a beam of 10 kv x-rays.

[4] Generously supplied by E. C. Reifenstein, Jr., M.D., of the Squibb Institute of Medical Research.

Principles

The calculations in this paper are based on the formulations of Bauer, Carlsson and Lindquist (2). These authors describe the movement of calcium and other constituents of the bone mineral between blood and bone as the resultant of three processes: (a) *accretion*, or the incorporation of elements in the non-exchangeable fraction of bone mineral; (b) *resorption*, or the solution of non-exchangeable bone mineral; this is equal to the difference between accretion and retention; and (c) *ion exchange*, or the movement of ions in and out of the exchangeable fraction of the bone mineral. According to this interpretation of tracer data, accretion and resorption represent two processes, both *irreversible* and working independently in opposite directions. Ion exchange implies a one-for-one *reversible* exchange of ions between blood and the exchangeable fraction of bone mineral.

The deposition of mineral in bone may be clearly visualized by autoradiography following administration of radiocalcium in the following locations: (a) the zone of calcification at the junction of the epiphyseal cartilage with the metaphysis; (b) subperiosteally in the shafts of the bones, where growth in diameter is occurring; and (c) sub-endosteally in the metaphyseal region forming the funnel which joins the region of epiphyseal-metaphyseal growth with the shaft (Fig. 5).

Calculations

The principles outlined in the foregoing section, as subjected to study by the use of radiocalcium, Ca^{45}, are formulated by Bauer, Carlsson and Lindquist (2) in the following equation:

$$Ca^{45}_{Obs} = Ca^{45}_{E} + Ca^{45}_{A} - Ca^{45}_{R} \qquad (1)$$

in which

Ca^{45}_{Obs} = total amount of Ca^{45} in calcified tissues
Ca^{45}_{E} = amount of Ca^{45} in the exchangeable fraction of the bone calcium
Ca^{45}_{A} = amount of Ca^{45} incorporated into the non-exchangeable fraction of the bone calcium through accretion
Ca^{45}_{R} = amount of Ca^{45} removed through resorption

Under the assumption that Ca^{45} and Ca^{40} are deposited in the bone mineral in the same proportions as those in which they are present in the plasma at any instant of time, Bauer, Carlsson and Lindquist derived the equation:

$$Ca^{45}_{A}(t) = A\int_{0}^{t} S(t)dt \qquad (2)$$

in which

A = rate of accretion of total Ca ($Ca^{40}+Ca^{45}$). This rate is assumed to remain constant for the duration of these experiments.
t = interval of time between administration of Ca^{45} and observation.
$\int_0^t S(t)dt$ = integrated specific activity of serum from time 0 to time t (see inset, Fig. 2). This is the notation currently used by Bauer, Carlsson and Lindquist (in press) and is equivalent to the quantity $T \times S_M$ in the notation previously used.

The *rate* of resorption of total calcium is calculated from the equation:

$$R = A - Ret \qquad (3)$$

in which

R = rate of resorption of total calcium
Ret = rate of retention of total Ca in bone, as calculated from the ash weights of the bone at intervals

Treatment of the Activity Data

The observed values of the specific activity of the serum were plotted and smooth curves were drawn to fit these values as closely as possible. The activity values, S(t), used in the calculations were taken from the curves (Fig. 2). Integration of the curves $\int_0^t S(t)dt$ was performed by estimating the area under each curve. Such estimates were made with a planimeter on large scale paper, and were verified by cutting out and weighing the areas under the curves.

The activity values, Ca^{45}_{Obs}, used for solving the equations and for construction of graphs were obtained from smooth curves drawn through plots of the observed values for the parts of the skeleton studied (Fig. 3). The accretion rate, A, and the value for exchangeable calcium, E, were calculated from the values thus obtained for Ca^{45}_{Obs} at 8, 16 and 24 hour intervals by substitution in the equations of Bauer, Carlsson and Lindquist (2). These values of A and E were then used for the calculation of Ca^{45}_{A} and Ca^{45}_{E}, by similar substitutions. Ca^{45}_{R} at each interval was obtained by substitution in Equation (1) above.

RESULTS

Influence of estrogen on growth.—The influence of estrogen on growth was observed by plotting the average increase in body weight (Fig. 1). Whereas in the control group the body weight increased by about 4.1 gm. per day, the increase for the estrogen-treated group was only about 2.0 gm. per day, corresponding to a reduction of approximately 50% in the increase in body weight. Similarly, Table 2 records the inhibiting effect of estrogen on the growth in length of the long bones. Over a period of 30 days, beginning from the age of 33 days, the average increase in the length of the femur was found to be 0.25 and 0.16 mm. per day for the control and estrogen-treated rats, respectively; in the tibia the corresponding figures were 0.25 and 0.15 mm. per day. The increase in length of the long bones was thus reduced by administration of estrogens by approximately 30%.

Roentgenograms revealed that within 2 weeks there was a demonstrable increase in the length of the core of spongy bone in the lower ends of the femurs and the upper ends of the tibias of the estrogen-treated rats. After 4 weeks the spongiosa of the upper ends of the tibias had attained a length of 2–3 mm., as compared with 1–2 mm. in the control rats (Fig. 4). The *microradiograms* (Fig. 5) confirmed the increase in length of the central part of the spongiosa of the estrogen-treated rats. The *autoradiograms*

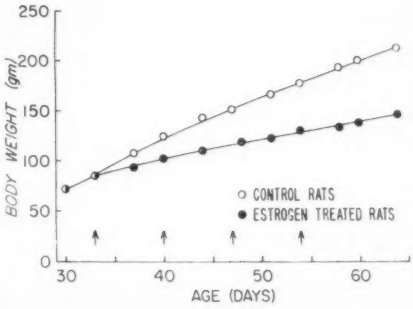

FIG. 1. Influence of estrogen on growth. Points represent averages for all rats of each group. Arrows indicate injections of estrogen or oil.

(Fig. 5) illustrate the similarity in the pattern of distribution of radiocalcium 1 hour after injection. At 8 days much of the activity in the spongiosa of the control rat had been removed by resorption, while it remained virtually unchanged in the estrogen-treated rat. In the two groups the growth at the epiphyseal line, as marked by the movement of radioactivity away from the epiphyseal plate, was approximately the same.

Kinetics of skeletal metabolism.—Radioactivity of the samples of blood serum is plotted in Figure 2. After the 1 hour interval the specific activity values S(t), for the estrogen-treated rats, were higher than for the controls, with the possible exception of the intervals after 2 days. Consequently the values for the integrated specific activity, $\int_0^t S(t)dt$, were higher for the estrogen-treated rats (inset, Fig. 2).

The observed activity values in the shafts and upper ends of the tibias are plotted in Figure 3, together with the curves for the separate fractions of the activity, from values derived as described above. In the shafts of the tibias it is seen that the curve for Ca^{45}_{Obs} coincides with the curve for $Ca^{45}_{A}+Ca^{45}_{E}$. For the upper ends of the tibias the curves for $Ca^{45}_{A}+Ca^{45}_{E}$ resemble the corresponding curves for the shafts; the curves for Ca^{45}_{Obs}, however, deviate at a significantly lower level. This deviation, which begins at about 24 hours, is attributed to loss of activity by resorption of bone, and the curves for Ca^{45}_{R} are plotted accordingly.

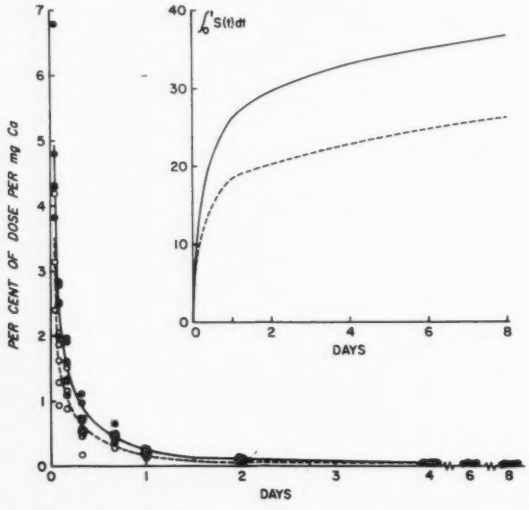

FIG. 2. Ca^{45} activity of blood serum in control rats (open symbols, interrupted line) and in estrogen-treated rats (solid symbols, continuous line). The values are expressed in per cent of dose/mg. of Ca. *Inset:* $\int_0^t S(t)dt$ = integrated specific activity of blood serum = area covered by the blood serum activity curves during intervals 0–t hours. (Controls, interrupted line; estrogen-treated, solid line.)

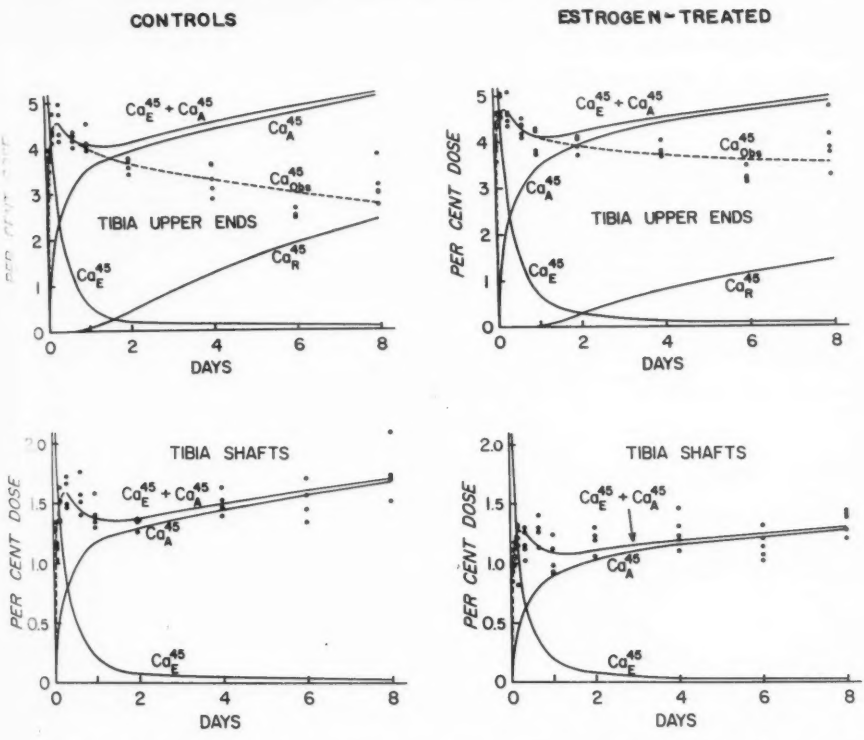

FIG. 3. Role of accretion, resorption and exchange reactions in the uptake and loss of Ca^{45} in the shafts and upper ends of the tibias. Dots indicate single observations (Ca^{45}_{Obs}); interrupted line (ends of tibias only) is smooth curve drawn through these points. In shafts curve for Ca^{45}_{Obs} is identical with sum of Ca^{45}_{E} (amount in exchangeable fraction) and Ca^{45}_{A} (amount added to non-exchangeable fraction by accretion). Ca^{45}_{R} (amount resorbed from non-exchangeable fraction) is obtained by subtracting Ca^{45}_{Obs} from $Ca^{45}_{E}+Ca^{45}_{A}$. Solid-line curves constructed from points calculated as described in text.

In the upper ends of the tibias a significant difference ($P<0.005$) is seen between the estrogen-treated and the control groups with respect to the *slopes* of the curves for Ca^{45}_{Obs}, the regression coefficients being -0.068 ± 0.026 and -0.180 ± 0.028, respectively, when calculated from the observed values from the 16 hour to the 8 day interval. This difference in Ca^{45}_{Obs} is reflected in Ca^{45}_{R}, where a corresponding difference is observed. In the shafts of the tibias, where resorption had no influence on the activity data during the experimental period, no significant difference in the *slopes* of the Ca^{45}_{Obs} curves is seen between the estrogen-treated and the control groups, the regression coefficients being 0.020 ± 0.010 and 0.040 ± 0.011, respectively, when calculated from the observed values from the 16 hour to the 8 day interval.

The values for the accretion rate, A, are presented in Tables 1 and 2. In all skeletal parts examined except incisors the absolute A values were significantly lower for the estrogen-treated rats than for the controls; in the femurs and upper ends of the tibias the values for the estrogen-treated animals were about two-thirds of those for the controls. In the shafts of the tibias the A value for the estrogen-treated group was about one-half that for the controls. In the incisors the A values were about the same for both groups, in agreement with the general experience that the incisors of rats continue to grow under conditions that markedly influence growth as a whole.

When the accretion rate for the tibias and femurs was studied in relation

TABLE 1. BONE SALT METABOLISM IN CONTROL AND ESTROGEN-TREATED YOUNG RATS

	Right femur		Both tibias, upper ends		Both tibias, shafts		Both tibias, lower ends		Four incisors	
	Control	Estrogen	Control	Estrogen	Control	Estrogen	Control	Estrogen	Control	Estrogen
Accretion rate (A) in mg. Ca hr.	0.153	0.100	0.193	0.133	0.064	0.035	0.032	0.019	0.085	0.079
Exchangeable Fraction (E) in mg. Ca	2.80	1.96	3.33	2.60	1.25	0.72	0.60	0.39	0.80	0.65
Ca content in mg.*	81.3	77.0	47.7	58.0	68.5	50.3	24.9	22.6	55.7	52.1
Retention rate (Ret) in mg. Ca / hr.†	0.067	0.059	0.046	0.059	0.047	0.025	0.021	0.016	0.032	0.029
Resorption rate (R) in mg. Ca hr.‡	0.086	0.041	0.127	0.074	0.017	0.010	0.011	0.003	0.053	0.050
Resorption Accretion (R A)	0.56	0.41	0.76	0.56	0.27	0.29	0.34	0.16	0.62	0.63

* These values are calculated from Table 3.
† Estimated from a value of 38% Ca in bone ash.
‡ In incisors these figures represent the attrition rate.

to their growth in length no difference was found between the controls and the estrogen-treated animals (Table 2). Similarly, the accretion rate for the estrogen-treated rats was reduced only in proportion to the lower rate of increase in body weight.

The *retention* rate (Ret) was derived from the increase in ash weight of the skeletal parts (Table 3). In all parts examined, including femurs, and sums of the segments of the tibias, but except for the upper ends of the tibias, the ash weights were higher for the controls than for the estrogen-treated rats, as was to be expected from the larger size and greater weights of the controls. The absolute values for the ash weights of the upper ends of the tibias, however, were higher in the estrogen-treated group, in spite of the smaller size of these animals. This difference is to be accounted for by the higher content of spongy bone resulting from the treatment with estrogen (cf. x-rays and microradiograms).

The two values for the net increase of bone mineral, as calculated from the increase during the period of the experiment and from that during the 8 days following administration of Ca^{45} were in good agreement. For the whole femurs and sums of the parts of the tibias almost identical values for

et increase were found. For the upper ends and shafts of the tibias there ere small differences, but these were not significant in the further calculaons. From these findings it is apparent that the retention rate of calcium as higher in the control group in all skeletal parts studied except for the pper ends of the tibias, where a higher value was found in the estrogenated group.

sorption.—The resorption rate (R) was calculated by subtracting the te of retention of Ca from the rate of accretion (Equation (3)). In all the skeletal tissues examined, except the incisors, the absolute figures for resorption (Table 1) are lower in the estrogen-treated group than in the controls. Moreover, it is seen that in the femurs, and in the upper and

TABLE 2. ACCRETION RATE RELATED TO GROWTH IN CONTROL AND ESTROGEN-TREATED YOUNG RATS

	Femur	Tibia
Average length in mm. at 33 days of age*	23.44	27.58
Average length in mm. at 63.8 days of age†		
Control	31.03	35.24
Estrogen	28.33	32.27
Increase in length in mm./day during the period of 30.8 days		
Control	0.246	0.249
Estrogen	0.159	0.152
Accretion rate (A) in mg. Ca/hr.‡		
Control	0.153	0.145
Estrogen	0.100	0.093
Accretion rate in relation to increase in length (A/L)		
Control	0.62	0.58
Estrogen	0.63	0.61

* These figures were obtained from one litter of 10 untreated rats.
† These figures were obtained from 100 rats treated with estrogen or oil from an age of 33 days; the average age was 63.8 days at time of sacrifice.
‡ These figures refer to one femur and one tibia. The figures for the tibias are the sums of the values of the different segments of tibias in Table 1.

lower ends of the tibias, the resorption rate had dropped considerably more than the corresponding accretion rates. Thus the figures for R/A in the femurs were 0.56 and 0.41 for the control and the estrogen-treated groups, respectively; for the upper ends of the tibias the corresponding figures were 0.76 and 0.56. In the shafts of the tibias and in the incisors the values for R/A were approximately the same for the experimental and the control groups.

Exchangeable calcium in the bones.—The amounts of exchangeable calcium (E) in the different skeletal parts studied were lower in the estrogen-treated than in the control groups (Table 1). In agreement with earlier investigations (2) there seems to be a correlation between E and A.

TABLE 3. ASH WEIGHTS OF CONTROL AND ESTROGEN-TREATED YOUNG RATS

	Right femur		Both tibias (sums of values of segments)		Both tibias, upper ends		Both tibias, shafts		Both tibias, Lower ends		Four incisors	
	Control	Estrogen	Control	Estrogen	Control	Estrogen	Control	Estrogen	Control	Estrogen	Control	Estrogen
Average ash wt. in mg. at 33 days of age*	81.9		150.5		47.0		79.9		23.6		80.9	
Average ash wt. in mg. at 63.8 days of age†	213.8	202.8	371.6	343.9	125.7	152.3	180.3	132.1	65.6	59.5	146.7	137.2
Increase in ash wt. in mg./day during period of 30.8 days	4.3	3.9	7.2	6.3	2.6	3.4	3.3	1.7	1.4	1.2	2.1	1.8
Increase in ash wt. in mg./day during period of 8 days following Ca^{45}‡	4.2	3.6	7.3	6.4	3.3	4.0	3.7	1.5	1.3	0.9	1.9	1.8
Retention rate (Ret) of Ca in mg./hr.§	0.067	0.059	0.112	0.101	0.046	0.059	0.047	0.025	0.021	0.016	0.032	0.029

* These figures were obtained from one litter of 10 untreated rats.

† These figures were obtained from 100 rats treated with estrogen or oil from an age of 33 days; the average age was 63.8 days at time of sacrifice.

‡ The ash weights of the rats in each of the two main groups were plotted against age (61–68 days) to obtain a graphic representation of the increase in bone ash.

§ Calculated from the mean of the two values for increase in ash weight. The calcium content in bone ash was taken as 38%.

DISCUSSION

Administration of estrogen to young rats causes a general reduction in the rate of growth of the skeleton and of the total body. The lower accretion values for the estrogen-treated animals as compared with the controls are accounted for by this effect; the difference in accretion values corresponded with the difference in rate of growth, whether evaluated by total body weight or by the length of a long bone.

In agreement with earlier studies on the effect of estrogens on the skeleton the trabeculae of the central part of the spongy bone in the estrogen-treated rats were longer than in the controls, as shown by x-rays and microradiograms; this effect is accounted for by inhibition of resorption in the spongiosa. In the shafts the inhibition of bone resorption was not more than could be accounted for by the general reduction in growth, consequently no effect was seen on the ratio R/A (Table 1). In the ends of the tibia, however, there was a more marked inhibition of bone resorption than could be accounted for by the reduction of growth, resulting in a decrease in the ratio R/A. In support of these findings the final slope of the observed activity curve of the upper ends of the tibias was significantly ($P < 0.005$) steeper in the controls than in the estrogen-treated rats, while no corresponding difference was seen in the shafts. The kinetic data thus agree with the morphologic observations.

Microscopic observation of the bones of estrogen-treated rats in earlier studies (1) gave the impression that inhibition of resorption of the trabeculae of bone in the center

FIG. 4. Roentgenograms of the tibias of control and estrogen-treated rats. Following continuous administration of estrogen the length of spongiosa in the metaphysis is increased and the density is indistinguishable from compact bone. Controls: *upper left*, male; *upper right*, female. Estrogen-treated rats: *lower left*, male; *lower right*, female. Estradiol n-valerate, 0.5 mg./wk. for 4 wks. ×1.5

of the spongiosa of the long bones was virtually complete. There was little or no thinning of the trabeculae in the primary spongiosa, and their extensions into the secondary spongiosa gave no evidence of shortening by resorption at the tips. From the present investigations, however, it appears that the total figure for resorption in the upper ends of the tibias was reduced by approximately 50%, indicating that resorption in this region was still active. Since the remodeling incident to growth was continuing, the external structure of the bones not being appreciably affected by estrogens, it appears that the resorption which participates in remodeling is not inhibited by this treatment.

The quantitative findings with respect to resorption of the spongiosa are also in agreement with the observations made by means of autoradiography. During an interval of 8 days following administration of Ca^{45} a large

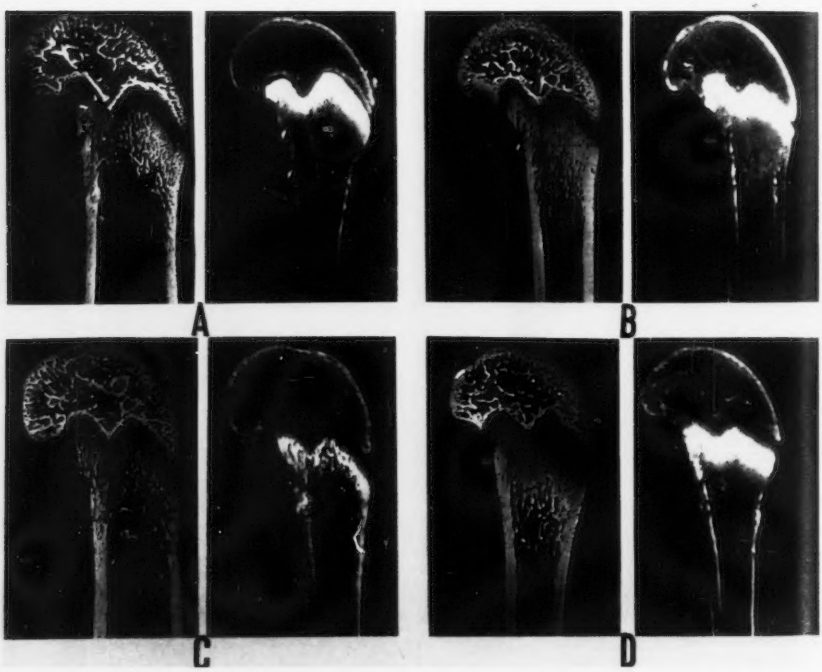

FIG. 5. Microradiograms (left) of sections and their autoradiograms (right) of control and estrogen-treated rats following administration of Ca^{45}. The sections were made from the distal ends of left femurs. Note the unresorbed spongiosa in the estrogen-treated rats. Compare with roentgenograms of Fig. 4. The uptake of Ca^{45} at 1 hour is identical in control and estrogen-treated rats. At 8 days there is retention of radioactivity in the estrogen-treated rat despite new endochondral growth below the cartilage plate, whereas in the control rat the corresponding activity has been removed by resorption of the spongiosa. *One hour post-injection: A*—Control; *B*—Estrogen-treated. *Eight day post-injection: C*—Control; *D*—Estrogen-treated. ×3.3

roportion of the radioactivity disappeared from the spongiosa of the ontrol animals, while that in the estrogen-treated animals was retained rtually without change (Fig. 5).

The observations in the present study provide additional evidence for e validity and usefulness of kinetic analysis, by the methods of Bauer, rlsson and Lindquist, in investigations of the metabolism of the bone neral.

REFERENCES

1. Budy, A. M., M. R. Urist and F. C. McLean: *Am. J. Path.* **28**: 1143. 1952.
2. Bauer, G. C. H., A. Carlsson and B. Lindquist: *Kungl. Fysiogr. Sällskap. Lund Förhandl.* **25**: 1. 1955.
3. Carlsson, A.: *Acta Pharmacol. et Toxicol.* 7, Suppl. **1**: 74 pp. 1951.
4. Clark, E. P. and J. B. Collip: *J. Biol. Chem.* **63**: 461. 1925.

INCREASED WATER PERMEABILITY OF THE BULLFROG (*RANA CATESBIANA*) BLADDER *IN VITRO* IN RESPONSE TO SYNTHETIC OXYTOCIN AND ARGININE VASOTOCIN AND TO NEUROHYPOPHYSIAL EXTRACTS FROM NON-MAMMALIAN VERTEBRATES[1]

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ABSTRACT

Neurohypophysial hormones accelerate the passage of water across the wall of the isolated bullfrog (*Rana catesbiana*) bladder *in vitro*. Net water movement may be in either direction depending on the existing osmotic gradient. Net sodium transport from mucosal to serosal surfaces has little influence on the magnitude or direction of water movement. The increased net water movement is considered, therefore, to be due to a change in water permeability, probably resulting from the dilatation of pores.

Oxytocin is more active than vasopressin in enhancing water permeability. The synthetic analogue arginine vasotocin is many times more active than oxytocin. The ratio of frog-bladder activity to oxytocic activity is high in neurohypophysial extracts from a lamprey, a teleost, a frog, a toad, a marine turtle, and the chicken. This suggests that arginine vasotocin may be a natural hormone in the neurohypophyses of many non-mammalian vertebrates.

DEHYDRATION or the injection of neurohypophysial hormones causes reabsorption of bladder water by frogs and toads (1, 2, 3). Water reabsorption appears to occur through the wall of the bladder itself (4). In *Rana pipiens* this response can be elicited with large doses of natural or synthetic oxytocin. Even larger doses of natural lysine vasopressin are necessary to causes significant water reabsorption in this species. Comparatively small doses of frog neurohypophysial extract are effective, however, indicating that the active principle in the frog neurohypophysis is neither oxytocin nor lysine vasopressin (4, 5).

Bentley (6) and Hays *et al.* (7) have studied the action of vasopressin on water movement across the bladder wall of *Bufo marinus in vitro*. The pres-

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[2] Early phases of this study were undertaken in the Department of Physiology, New York University College of Medicine, and during the author's tenure of a Lederle Medical Faculty Award (1955–1957).

nt paper deals with experiments done on the isolated bullfrog (*Rana catesbiana*) bladder *in vitro*. The influences of osmotic gradient and net sodium transport on water movement have been investigated. The relative potencies of synthetic and natural neurohypophysial hormones and synthetic analogues in increasing net water movement across the bullfrog bladder wall have also been investigated. This information has been used for the preliminary identification of the pressor-antidiuretic hormone in certain nonmammalian vertebrates.

METHODS

Large male *Rana catesbiana* from Louisiana were anesthetized by immersion in 0.2% MS222 (Tricaine methanesulfonate, Sandoz). The bladders were dissected free and removed intact. Glass cannulae were tied into the necks of the bladders and each was suspended so that the bladder was submerged in an individual chamber containing 50 ml. of Ringer's solution of the following composition: NaCl 80, KCl 2.5, $CaCl_2$ 1.8, $NaHCO_3$ 24 mM/l, NaH_2PO_4 0.1 and glucose 0.2 gm./l. This outside solution was gassed with 5% CO_2 in oxygen and had a pH of 7.4 and a total osmolarity of approximately 200 mOs/l.

Ten ml. of solution was placed inside each bladder. The composition of the inside solution varied with the experiment. In the initial experiments the bladder contents were removed and measured at appropriate intervals. In later experiments bladder volume was estimated by weighing the bladders to within 10 mg. at 15 minute intervals after brief blotting on a smooth surface. In the initial experiments bovine albumin labelled with T-1824 was included in the inside solution as a means of detecting gross leakage. This later proved unnecessary since the water movement during control periods served as a sensitive indication of the integrity of the bladder wall.

Osmolarity was measured by a semi-micro modification of the Johlin (8) method using thermistors (9) and a rotary stirrer (10) or with a Fiske osmometer. Sodium concentrations were determined by an internal standard flame photometer.

Oxytocic activity was determined by Dr. R. A. Munsick utilizing his modification of the Holton (11) rat uterus method. Uteri were suspended in a van Dyke-Hastings solution without magnesium (12).

RESULTS

The effect of osmotic gradient on net water movement

Isolated bladders were filled with a solution containing bovine albumin labelled with T-1824, creatinine, and inulin. The total osmolarity of the inside solution was 9–27 mOs/l. Under these circumstances whole neurohypophysial extract (Pituitrin, Parke, Davis & Co.) or synthetic oxytocin,[3] 100mU./ml. in the outside solution, causes an acceleration of the rate of decrease in the measured volume of the bladders and a corresponding increase in the concentrations of T-1824, creatinine, and inulin in the inside solutions (Table 1). These hormones appear to accelerate the rate of water loss from a hypotonic inside solution into the surrounding Ringer's solution without a concomitant loss of solutes.

[3] Kindly supplied by Dr. Vincent du Vigneaud of Cornell Medical College.

If bladders are filled with a similar solution to which sucrose has been added to bring the total osmolarity to approximately twice that of the surrounding Ringer's solution then addition of neurohypophysial extract to the Ringer's accelerates the rate at which water enters the bladders (Table 1) and dilutes the contained solutes. Recovery of normal water permeability appeared to be delayed in these bladders. Water movement returned to 5.9–8.9 μl./cm.^{2}/hr., however, in 3 of the 4 bladders $1\frac{1}{2}$ to 3 hours after the end of exposure to neurohypophysial hormone.

It appears, therefore, that neurohypophysial hormones can augment the water passage in *either* direction across the wall of the bullfrog bladder.

TABLE 1. THE ACTION OF NEUROHYPOPHYSIAL EXTRACT (PITUITRIN) ON NET WATER MOVEMENT AND SOLUTE CONCENTRATION IN THE ISOLATED BULLFROG BLADDER

Time, hours	Dose of pituitrin	Ratio: $\frac{\text{final concentration}}{\text{initial concentration}}$			Measured net water movement, μl./cm^2./hr.*
		T-1824	Creatinine	Total osmolarity	
Hypotonic bladder solution. 4 bladders					
0–1.5	None	1.01	1.00	1.14	− 4.0±2.6†
1.5–3	100 mU./ml.	1.59	1.50	1.87	−96 ±11
3–4.5	None	1.09	1.05	1.28	− 6.1±2.1
4.5–6	None	1.04	1.01	1.13	− 3.5±1.8
Hypertonic bladder solution. 4 bladders					
0–1.5	None	0.98	0.97	0.98	+ 6.8±2.8
1.5–3	100 mU /ml	0.89	0.90	0.90	+31 ±1.8
3–4.5	None	0.91	0.90	0.91	+26 ±2.8
4.5–6	None	0.94	0.92	0.95	+14 ±6.0

* Positive figures indicate net water movement from serosal to mucosal surfaces, negative figures indicate movement in the reverse direction.
† Standard error.

The hormone concentration used in these experiments, 100 mU./ml., causes a maximal response if applied to the serosal surface of the bladder. 200 mU./ml. applied to the mucosal side is without appreciable effect on water movement. Leaf *et al.* (13) have observed that neurohypophysial hormone applied to the mucosal surface of the toad bladder does not alter sodium transport as it does if applied to the serosal surface.

The relationship between net water movement and net sodium transport

Leaf, Anderson and Page (13) have reported that neurohypophysial hormones increase the rate of active sodium transport from mucosal to serosal surfaces of the isolated toad bladder. Since Capraro and Garampi (14) and Bentley (15) have postulated that the influence of neurohypophysial hormones on water movement across the frog or toad skin may be secondary to sodium transport we undertook to investigate the relationship in the

isolated bullfrog bladder. An experiment was designed in which the inside bladder solution contained an initial concentration of 10 mEq/l. of NaCl. This concentration is well in excess of that usually encountered in ureteral urine from bullfrogs even during intense antidiuresis caused by neurohypophysial hormone administration (3). The outside solution was unchanged but the total osmolarity of the inside solution, and, therefore, the effective osmotic gradient, was altered by the addition of sucrose. The net transport of sodium was determined by measurement of changes in sodium concentration and volume of the inside solutions.

A typical experiment of this type is illustrated in Figure 1. Oxytocin

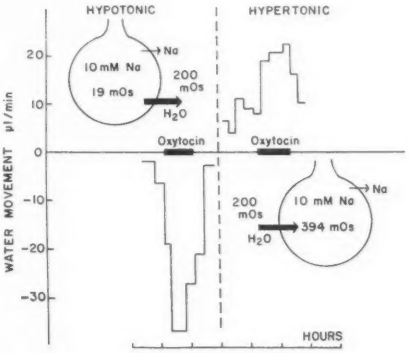

FIG. 1. The effect of 100 mU./ml. of synthetic oxytocin on the movement of water across the wall of a frog bladder containing a hypotonic solution and, subsequently, a hypertonic solution. The sodium concentration in both inside solutions was 10 mEq/l. The outside solution had an osmotic concentration of 200 mOs/l. in both cases.

(Syntocinon, Sandoz[4]), 100 mU./ml., augmented water movement out of the bladder containing a hypotonic solution, and into the bladder when the osmotic gradient was reversed. Net sodium transport was usually from inside to outside whether or not oxytocin was present and irrespective of the direction of water movement. Oxytocin did not significantly alter net sodium transport in the majority of trials (Fig. 2). Total sodium flux in either direction could not be ascertained from these experiments. Alterations in sodium influx or outflux of sufficient magnitude to explain the change in the direction of water movement could probably not occur without measurable alteration in the direction of net sodium transport.

The enhancement of net sodium transport by oxytocin in the bullfrog bladder was, at best, inconsistent. The addition of 10 mEq./l. of NaCl to the bladder solution did not noticeably increase the movement of water (Fig. 2) from inside to outside as would be expected if net sodium transport were the driving force for water movement after neurohypophysial hormone administration. The experiments summarized in Figure 2 appear most consistent with the hypothesis that water movement in response to neuro-

[4] Kindly supplied by Dr. R. Bircher, Medical Department, Sandoz Pharmaceuticals, Hanover, N. J.

hypophysial hormones depends largely upon the osmotic gradient. Sodium transport is involved to a very limited extent if at all.

The response of the isolated bladder to neurohypophysial hormones and their analogues

The frog bladder *in situ* responds to natural or synthetic oxytocin but is relatively less sensitive to lysine vasopressin (4). Compared to oxytocin frog neurohypophysial extract is much more active on the bladder than can be explained by its oxytocic activity. Similar observations have been made on the responses of the frog skin (16) and kidneys (5). Quantitation of this relationship has been difficult in *in vivo* preparations. The isolated bladder, however, offers a preparation in which more accurate estimation of relative potency is possible.

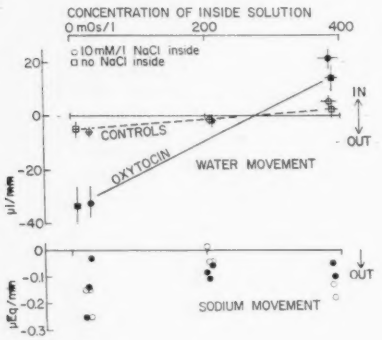

FIG. 2. Net sodium and water movement across the frog bladder membrane in the absence (open figures) and the presence (closed figures) of 100 mU./ml. of synthetic oxytocin. The total osmolarity of the inside solutions next to the mucosal surface was adjusted to the values shown by the addition of sucrose. The curves drawn are straight lines connecting the mean points for water movement for bladders containing no added sodium. Vertical and horizontal lines through the points for water movement indicate the standard deviations of these points, each of which represents the mean of at least four bladders.

The isolated bladder is filled with 10 ml. of water, weighed repeatedly to ensure against leakage, and then the hormone to be assayed is added to the outside bath. The weight lost during the interval 15 to 45 minutes after hormone addition is taken as the response. The bladder is then washed and refilled to its initial volume and the outside solution is changed. Sufficient time is allowed for water movement to return to a normal resting rate (less than 10 µl./min.) between successive administrations of hormone. In the course of a working day three or four hormone periods can be observed on each bladder.

There is an approximately linear regression of response against log dose in the submaximal range with this preparation. Since concentrations over 60 mU./ml. give maximal or close to maximal responses doses of oxytocin of 5–40 mU./ml. are used. There is great variability between individual bladders, however, making it necessary to bracket unknown injections with standards or to use a four-point assay design. Relative potency in either case is calculated on the assumption of a linear regression of response on

log dose. A frog-bladder unit is defined as an amount of extract or hormone required to cause a response equivalent to that produced by one unit of USP Posterior Pituitary Reference Standard. Synthetic oxytocin (Syntocinon, Sandoz[4]) that had been restandardized in this laboratory by rat uterus assay against the USP Standard was used as a working standard for the frog bladder assay.

Relative to oxytocin, the potency of a single preparation from a non-mammalian source varied if assays were performed on several bladders. This is probably due to the fact that the frog-bladder activity of such an extract resides in a peptide other than oxytocin. There is little reason to expect that the sensitivities of a series of bladders to both oxytocin and the unknown should vary in a strictly parallel fashion.

A typical four-point assay on a frog bladder is illustrated in Figure 3.

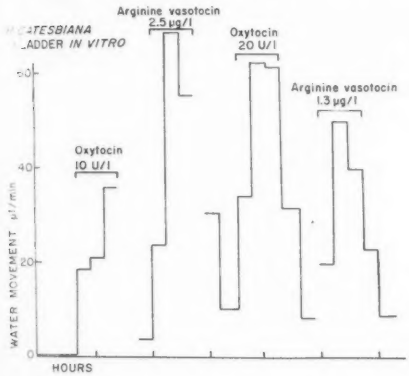

FIG. 3. A four-point assay of arginine vasotocin against synthetic oxytocin on a bullfrog bladder *in vitro*. 20 U. of oxytocin would represent approximately 55 µg. of the pure peptide.

The frog bladder assay was applied in a preliminary investigation of the relationship between chemical structure and activity on the frog bladder. Highly purified natural lysine and arginine vasopressins and synthetic oxytocin, 2-phenylalanine oxytocin, "oxypressin" (3-phenylalanine oxytocin) and arginine "vasotocin" (8-arginine oxytocin) have been generously made available for pharmacological studies in this Department by Dr. Vincent du Vigneaud and his coworkers of the Department of Biochemistry, Cornell Medical College. The relative potencies of these hormones and their analogues on many different assays have been studied in detail by van Dyke *et al.* (17). These preparations, and a solution of 3-valine oxytocin (Sandoz[4]) were assayed for frog bladder activity. The results of such assays are shown in Table 2. For oxytocin the ratio of frog-bladder activity to oxytocic activity (rat uterus assay) is, by definition, 1.0. Substitutions in the oxytocin ring at positions 2 or 3 appear to reduce the absolute oxytocic and frog-bladder potencies in a roughly parallel manner. This is true even when lysine or arginine is also substituted in the side chain, as in the vasopressins. If, however, the oxytocin ring remains intact and arginine is sub-

TABLE 2. RELATIONSHIP BETWEEN CHEMICAL STRUCTURE, OXYTOCIC AND FROG-BLADDER ACTIVITIES OF NEUROHYPOPHYSIAL HORMONES AND SOME SYNTHETIC ANALOGUES

Oxytocin

$$CyS \cdot Tyr \cdot Ileu \cdot Glu(NH_2) \cdot Asp(NH_2) \cdot CyS \cdot Pro \cdot Leu \cdot Gly(NH_2)$$

(Tyr: 2; Ileu: 3; CyS: 8)

Preparation	Substitution on oxytocin	Activities, U./mg.		Ratio bladder/uterus
		Rat uterus	Frog bladder	
Oxytocin	None	360	360	1.0
2-Phenylalanine oxytocin	2-Phe	30	18	0.6
Arginine vasotocin	8-Arg	37	7800	210
Oxypressin	3-Phe	16	<20	<1.2
Arginine vasopressin	3-Phe,8-Arg	15	21	1.4
Lysine vasopressin	3-Phe,8-Lys	18	<5	<0.3
Valyl oxytocin	3-Val	1.8 U./ml.	0.4 U./ml.	0.22

stituted for leucine in the penultimate position of the side chain, the frog-bladder activity is increased about 20 times. The oxytocic activity of this analogue, arginine vasotocin, is about 1/10 that of oxytocin. Arginine vasotocin has, therefore, about 200 times the frog-bladder activity that one would predict from its oxytocic activity. Of the analogues studied to date it is the only one in which there is a frog-bladder/oxytocic activity ratio clearly above unity.

Arginine vasotocin was synthesized by Katsoyannis and du Vigneaud (18). It has other interesting pharmacological characteristics. It is far more active in causing contraction of the hen and turtle oviducts or in producing antidiuresis in the hen than is oxytocin or the vasopressins (19).

Frog bladder activity in nonmammalian neurohypophysial extracts

Arginine vasotocin has been tentatively identified in chicken neurohypophysial extracts (19). It was of interest, therefore, to observe the response of the frog bladder to chicken extract. The activity of such a preparation on the bladder, relative to its oxytocic activity, is exactly what one would predict if it contained arginine vasotocin (Fig. 4).

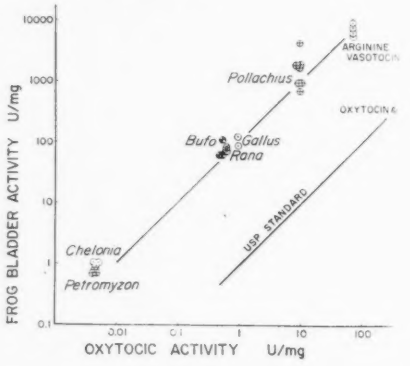

FIG. 4. The relationship between frog-bladder activity and oxytocic activity for neurohypophysial extracts from nonmammalian vertebrates. The lower curve represents, by definition, the activities of USP Standard. The upper curve is the line on which neurohypophysial extracts would fall if their frog-bladder activity were due to arginine vasotocin. Each point represents a separate frog bladder assay.

Heller (16, 20) observed that extracts from neurohypophyses of the cod, frog, snake and pigeon contain much more frog water-balance activity than can be accounted for by their oxytocic content. The frog bladder assay offers a means for comparing extracts from nonmammalian vertebrates to arginine vasotocin. As shown in Figure 4 extracts from marine lampreys (*Petromyzon marinus*), pollack (*Pollachius virens*[5]), toads (*Bufo americanus*), bullfrogs (*R. catesbiana*) and green turtles (*Chelonia mydas*) were compared to oxytocin by the frog bladder assay. It can be seen that in each case the ratio of frog bladder activity to oxytocic activity is approximately that which would be predicted on the presumption that these extracts contained arginine vasotocin. Although there is evidence that some or all of these extracts may contain oxytocin as well (19, 21) the specificity of the frog bladder assay is such that the error introduced by oxytocin would be minimal.

DISCUSSION

The observation that neurohypophysial hormones increase the permeability of the frog bladder wall to water essentially independently of their action on net sodium transport is consistent with the findings of Bentley (6) using the toad bladder. It also agrees with our earlier hypothesis (3, 4, 5) that neurohypophysial hormones dilate pores in the bladder wall as they do in the skin of anurans (23, 24). This has recently received strong support from Hays *et al.* (7) who have demonstrated increased permeability of the toad bladder to urea and acetamide in response to neurohypophysial hormone. It thus seems probable that the action of neurohypophysial hormones on the skin and bladder of anurans is dual in nature. They may increase sodium transport, and, in so doing, increase water movement (14, 15). They can also change the permeability of the membranes to water and allow a more rapid osmotic flow through minute pores. This second action appears to be largely independent of the existence or direction of net sodium transport in the bullfrog bladder.

There is reason to believe that the antidiuretic effects of neurohypophysial hormones result from a similar action on the water permeability of the distal renal tubule (5, 25). The dissociation of neurohypophysial action on water movement from direct dependence on sodium transport is important if meaningful comparisons are to be made between antidiuretic responses by the distal tubule and responses observed in the skins and bladders of frogs and toads.

The marked specificity of the response of the bullfrog bladder to arginine vasotocin offers a new tool for exploring the nature of neurohypophysial hormones of nonmammalian vertebrates. The information presented in this paper and collaborative evidence from studies in progress (21) are

[5] Partially purified material identified as fraction F65F-64B by Wilhelmi, Pickford and Sawyer (22). The starting material was whole frozen pituitaries from a variety of marine teleosts but predominantly from pollack.

consistent with the hypothesis that arginine vasotocin is a widely distributed natural neurohypophysial hormone among nonmammalian vertebrates. Direct proof of the presence of arginine vasotocin in these vertebrates must, however, await its isolation and positive chemical identification.

REFERENCES

1. STEEN, W. B.: *Anat. Rec.* **43**: 215. 1929.
2. EWER, R. B.: *J. Exper. Biol.* **29**: 173. 1952.
3. SAWYER, W. H.: *Mem. Soc. Endocrinol.* **5**: 44. 1955.
4. SAWYER, W. H. AND R. M. SCHISGALL: *Am. J. Physiol.* **187**: 312. 1956.
5. SAWYER, W. H.: *Proc. 8th Colston Res. Symp.* 1957. p. 171.
6. BENTLEY, P. J.: *J. Endocrinol.* **17**: 201. 1958.
7. HAYS, R. M., LAMDIN, R. H. MAFFLY AND A. LEAF: *Fed. Proc.* **18**: 66, 1959.
8. JOHLIN, J. M.: *J. Biol. Chem.* **91**: 551. 1931.
9. WESSON, L. G., JR.: *Methods in Med. Res.* **5**: 175. 1952.
10. BOWMAN, R. L., H. V. TRANTHAM AND P. A. CAULFIELD: *J. Lab. & Clin. Med.* 43: 310. 1954.
11. HOLTON, P.: *Brit. J. Pharmacol.* **3**: 328, 1948.
12. VAN DYKE, H. B. AND A. B. HASTINGS: *Am. J. Physiol.* **83**: 563. 1928.
13. LEAF, A., J. ANDERSON AND L. B. PAGE: *J. Gen. Physiol.* **41**: 657. 1958.
14. CAPRARO, V. AND M. L. GARAMPI: *Mem. Soc. Endocrinol.* **5**: 60. 1955.
15. BENTLEY, P. J.: *J. Endocrinol.* **16**: 126. 1957.
16. HELLER, H.: *J. Physiol.* **100**: 124. 1941.
17. VAN DYKE, H. B., R. A. MUNSICK AND W. H. SAWYER: *Fed. Proc.* **18**: 163. 1959.
18. KATSOYANNIS, P. G. AND V. DU VIGNEAUD: *J. Biol. Chem.* **233**: 1352. 1958.
19. MUNSICK, R. A., W. H. SAWYER AND H. B. VAN DYKE: *Fed. Proc.* **18**: 110. 1959.
20. HELLER, H.: *J. Physiol.* **101**: 317. 1942.
21. SAWYER, W. H., R. A. MUNSICK AND H. B. VAN DYKE: *Nature*, in press.
22. WILHELMI, A. E., G. E. PICKFORD AND W. H. SAWYER: *Endocrinology* **57**: 243. 1955.
23. KOEFOED-JOHNSEN, V. AND H. H. USSING: *Acta Physiol. Scandinav.* **28**: 60. 1953.
24. ANDERSEN, B. AND H. H. USSING: *Acta Physiol. Scandinav.* **39**: 228. 1957.
25. WIRZ, H.: *Helv. Physiol. Acta.* **14**: 353. 1956.

INFLUENCE OF STILBESTROL ON THE IMMATURE RAT OVARY AND ITS RESPONSE TO GONADOTROPHIN[1]

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ABSTRACT

The ovarian response to stilbestrol and to Armour's pituitary gonadotrophin (LH) with or without stilbestrol priming was studied in both intact and hypophysectomized immature rats. In the doses used (1 mg. daily for two days) stilbestrol caused an increase in ovarian weight in intact rats and retarded the atrophy after hypophysectomy. Stilbestrol priming markedly augmented the ovarian response to gonadotrophin (LH) in both intact and hypophysectomized rats. Using a consistent dose of LH (0.5 mg.) but priming doses of stilbestrol ranging from 0.002 mg. to 2.0 mg. showed that the larger doses of stilbestrol augmented the ovarian response. Stilbestrol augments the ovarian response by making many follicles responsive to gonadotrophin. The similarity of responses in intact and hypophysectomized rats indicates that large doses of stilbestrol directly influence the ovary.

MAZER, Israel, and Alpers (1) found that estrogen (500 R.U. daily for seven days) caused an increase in ovarian weight in infantile rats. This estrogen treatment also favored the formation of corpora lutea, so it was assumed that the estrogen action was mediated bythe pituitary. Pellets of diethyl-stilbestrol inserted subcutaneously into hypophysectomized rats caused an increase in ovarian weight (2). The presence of the stilbestrol pellet also markedly augmented the ovarian response to injections of human chorionic gonadotrophin (HCG). Williams (3) noted that stilbestrol pellets inserted two days after hypophysectomy prevented or retarded the atrophy of the ovaries and augmented their response to pregnant mare's serum hormone (PMS). He also noted that the stilbestrol did not produce increased ovarian weights in intact rats. Simpson, Evans, Fraenkel-Conrat, and Li (4) found that stilbestrol (1 mg. daily for eight days) caused a marked increase in ovarian weight in hypophysectomized rats. After this estrogen treatment their rats exhibited an augmented response to HCG and to FSH but not to ICSH. Payne and Hellbaum (5) found that doses of 1 mg. per day for four days were more effective in causing increased ovarian weights in hypophysectomized rats than were smaller doses of stilbestrol. Payne and Runser (6) found that when stilbestrol was given simultaneously with a pituitary extract (Searle-Gonadophysin)

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there was an augmented response but not when estrogen was given simultaneously with HCG.

These studies on the augmenting effect of stilbestrol in hypophysectomized rats indicated that large doses of stilbestrol had a stimulating effect on the ovary. Since this seemed to be a direct effect on the ovary, it seemed probable that a similar effect should be demonstrable in the intact rat. The present experiments were conducted to compare the ovarian response to gonadotrophins alone and after stilbestrol priming in both intact and hypophysectomized rats.

MATERIAL AND METHODS

Immature rats weighing 50 to 60 gm. were purchased from Sprague-Dawley, Incorporated. The hypophysectomized rats were Sprague-Dawley rats purchased through Hormone Assay Laboratories, Inc., Chicago, and weighed 50 to 60 gm. when received two days postoperatively. On arrival in the laboratory, the hypophysectomized rats were given 2.5 mg. of cortisone acetate subcutaneously and sugar was added to their drinking water for the first day. After that all rats were maintained on tap water and laboratory chow *ad libitum*. The rats were weighed and sorted into test groups of comparable initial weights.

Stilbestrol doses in 0.1 cc. peanut oil were given subcutaneously for two days. Groups of stilbestrol-treated rats were caged separately. The gonadotrophin was Armour's Pituitary Gonadotrophin LH (Lot No. R377242H).[2] Sterile saline (10 cc.) was added to the ampule to dissolve the 100 mg. and this was used as a stock solution. The amount needed for each experiment was withdrawn from the stock bottle which was then stored in the deep freeze until the next test. Test solutions were diluted so that the total dose for each rat was in 2.5 cc. of saline. Five doses (0.5 cc. each) were given each rat over a three-day interval, the first dose being 24 hours after the second injection of stilbestrol in the primed rats. At the end of the test (72 hours after the first injection of LH or 120 hours after the first priming dose of stilbestrol) the rats were asphyxiated with natural gas and weighed. Hypophysectomized rats that had gained in weight were checked for pituitary remnants and discarded if incompletely hypophysectomized. The ovaries were excised, examined, weighed on a Roller-Smith torsion balance, and fixed in Bouin's solution. The condition of the uterus was noted at autopsy but the doses of stilbestrol were such that no quantitation was considered. The ovaries were sectioned at five microns and stained with hematoxylin and eosin. The number of rats in this study totaled 88 hypophysectomized and 163 intact.

RESULTS

The average ovarian weights in hypophysectomized rats after various doses of gonadotrophin are depicted in Figure 1. The 2 mg. priming dose of stilbestrol probably maintained the ovarian weight (av. 14.0 mg.) at about the preoperative level and the 0.2 mg. dose of LH had little additional effect on the estrogen-primed ovaries. Higher doses of LH were more effective in causing ovarian weight gain in the estrogen-treated rats. These findings are in accord with previous reports.

[2] Although this extract was designated LH it was probably a mixture of gonadotrophins. In exploratory tests Armour's FSH was added to this "LH" and then injected into immature rats. The added FSH failed to augment the ovarian weight response so it is presumed that the original "LH" contained optimal quantities of FSH.

The average ovarian weights of intact rats after various doses of LH are depicted in Figure 2. Except for the ovaries being heavier, the augmented responses of the intact estrogen-primed rats are similar to those noted for hypophysectomized rats. Why this augmentation should become so marked at doses of 0.5 mg. and higher is not evident.

Since previous attempts to demonstrate augmentation by estrogen priming had been unsuccessful with small doses of estrogen it became necessary to test the influence of stilbestrol dosage on the augmentation of ovarian responses in both intact and hypophysectomized rats. The 0.5 mg. dose of

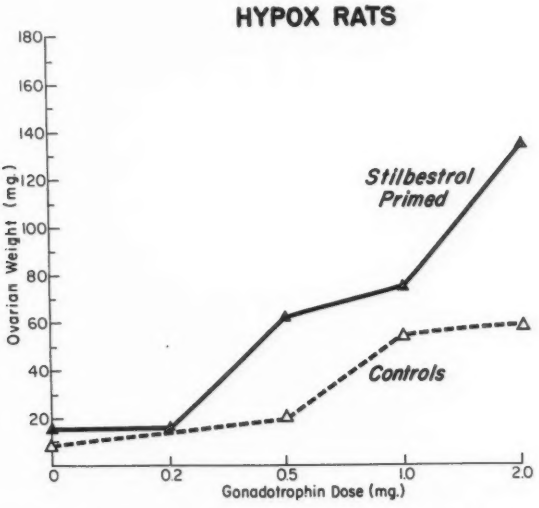

FIG. 1. Ovarian weight responses of hypophysectomized rats to increasing doses of gonadotrophin. Stilbestrol had been given to one group of rats (1 mg. per day for 2 days) prior to starting the gonadotrophin injections. Ovarian weights are averages for 4 or 5 rats at each dose group.

LH had been effective in previous tests so it was selected as the standard dose. Stilbestrol doses were varied from 0.002 mg. to 2.0 mg. The results are shown in Figure 3. Over this dose range of stilbestrol it is apparent that the larger doses have a greater augmenting effect. Further testing is necessary to determine whether the crossover of the curves at 0.2 mg. (Fig. 3) is significant. Doses of stilbestrol larger than 1 mg. per day tend to be toxic so there were no attempts to extend this dose response curve.

Injection of a single priming dose 24 hours before starting the gonadotrophin was less effective than the two doses of stilbestrol and 48 hours of priming. Obviously a series of studies are now indicated to determine the optimal time and dose for maximal augmentation.

Histologically the augmented ovarian response is associated with increased numbers of vesicular follicles. In the intact rat the 0.5 mg. dose of

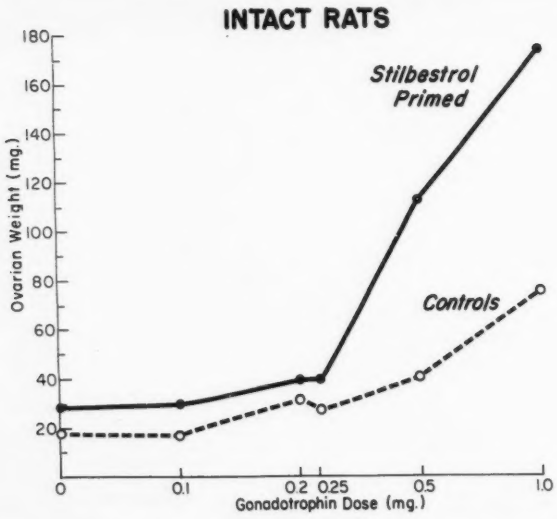

FIG. 2. Ovarian weight responses of intact immature rats to increasing doses of gonadotrophin. Stilbestrol had been given to one group of rats (1 mg. per day for 2 days) prior to starting gonadotrophin injections. Ovarian weights are averages for 6 to 12 rats at each dose group.

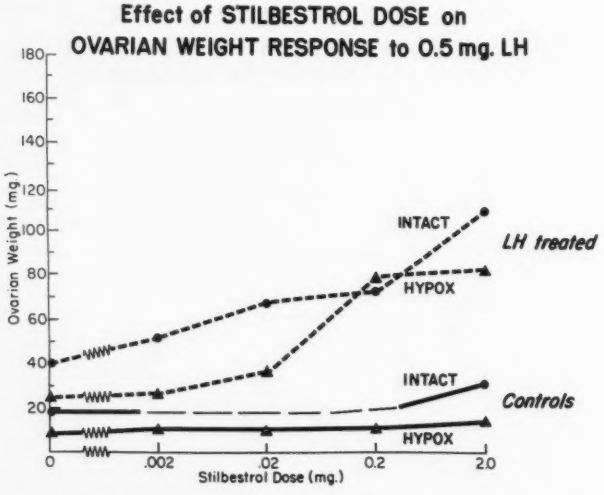

FIG. 3. Ovarian weight responses of intact and hypophysectomized rats to increasing doses of stilbestrol. A standard dose of gonadotrophin (0.5 mg.) was given to two groups of rats. Five rats at each dose in hypophysectomized groups and 3 to 12 intact rats at each dose.

LH stimulated a few follicles to an advanced vesicular stage, luteinized several follicles in each ovary, and caused hypertrophy of the interstitial tissue (Fig. 4). The estrogen primed controls had an increased number of medium-sized prevesicular follicles (Fig. 5). The estrogen primed, LH treated ovaries were composed of many vesicular follicles (Fig. 6). The estrogen pretreatment seemed to reduce the incidence of luteinized follicles and in some way conditioned many follicles to respond in a way usually considered the typical response to FSH extracts. The estrogen controls

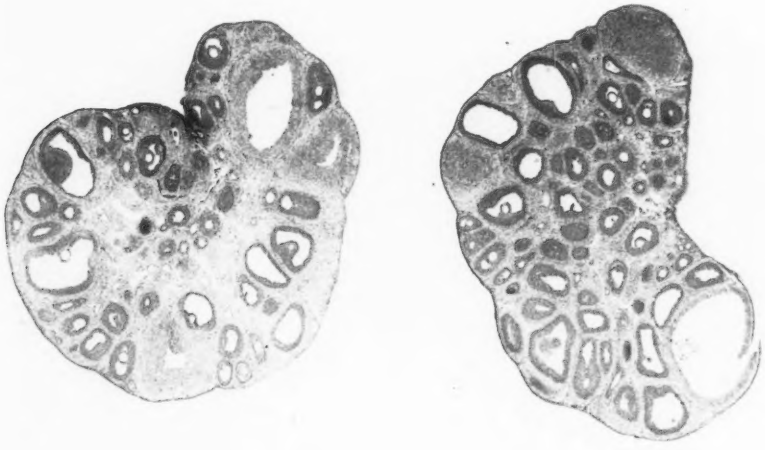

FIG. 4. Cross-section of a pair of ovaries from intact rat after 72 hours treatment with gonadotrophin (0.5 mg.). Several large cystic follicles and luteinized follicles and hypertrophied interstitial tissue. Average wt. per pair 40 mg. ×25.

killed at 48 hours showed very little histological alteration which would indicate any reasons for the augmented response. The controls killed at 120 hours showed more medium-sized follicles and frequently the peculiar atresia described by Simpson *et al.*

DISCUSSION

These experiments confirm the earlier reports that stilbestrol maintains or stimulates ovaries in hypophysectomized rats and also that similar effects occur in intact rats. The augmented weight response of ovaries to gonadotrophin in estrogen-primed rats is similar in hypophysectomized and intact rats. The stimulating effect on ovarian weight and also the augmented response increases with the dose of stilbestrol. It thus becomes apparent that the large doses must have a direct action upon the ovary.

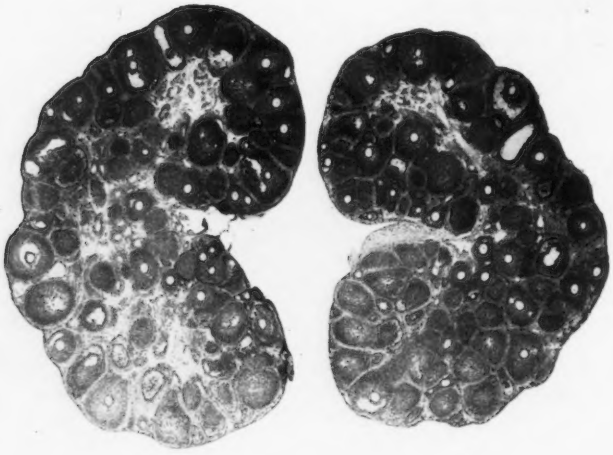

FIG. 5. Cross-section of a pair of ovaries from intact rat 120 hours after first of two injections of stilbestrol. Active granulosal proliferation in many medium sized prevesicular follicles. Average wt. per pair 29 mg. ×25.

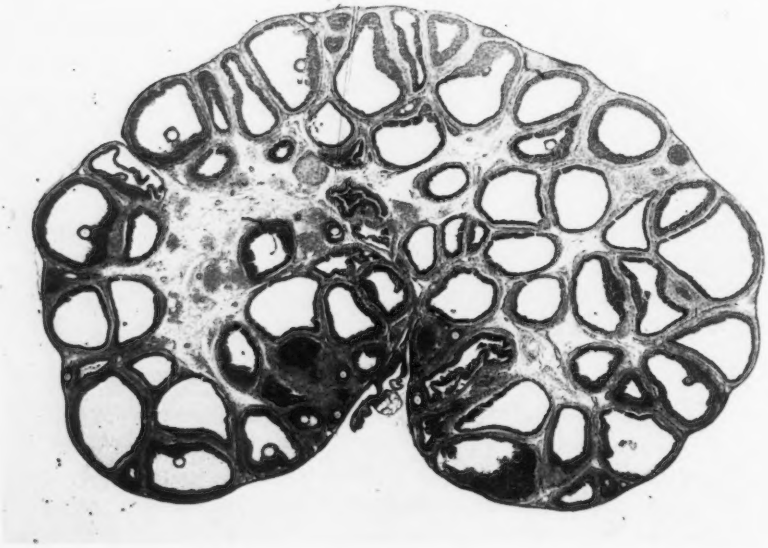

FIG. 6. Cross-section of an ovary from intact rat treated with 2 mg. of stilbestrol and then given 72 hour treatment with gonadotrophin (0.5 mg.). Many large vesicular and cystic follicles. Average wt. per pair, 112 mg. ×25.

The findings are somewhat at variance with current concepts but the differences can be rationalized. Moore and Price (7) stated that gonadal hormones have no direct effect on the gonads. However, their experiments were done with estrogen in doses of 12 rat units per day (ca. .012 mg.) for 10 days whereas the present experiments utilized 1 mg. (ca. 2500 rat units) per day for only two days. Pencharz (2) noted that 0.04 to 0.06 mg. per day of estradiol dipropionate was not as effective as 0.13 to 0.17 mg. of stilbestrol. In our experiments 0.1 mg. of stilbestrol was not as effective as 1.0 mg. It can be assumed that these large doses get effective amounts of estrogen into the ovaries.

A number of earlier attempts to obtain augmentation of ovarian weight responses to gonadotrophins were unsuccessful. Bradbury (8) showed that small doses of estrogen caused a release of gonadotrophin from the pituitary and caused a slight increase in ovarian weight but no augmentation to exogenous gonadotrophin. Paesi (9) confirmed these findings for doses of estrogen up to 0.1 mg. per day and argued that augmentation should not be expected. These experiments like those of Moore and Price were done with estrogen in dose ranges that influence gonadotrophic activity of the pituitary but have no appreciable direct action on the ovary.

Unfortunately, neither Pencharz nor Williams specified the duration of estrogen priming in their experiments. Simpson *et al.* (4) injected estrogen for eight days before giving any gonadotrophin and found marked augmentation when HCG was given. Paesi (10) reviewed the conditions whereby estrogen-priming produced augmented responses to gonadotrophins, the so-called Collip Effect. He treated immature rats with estrone (20 μg. per day for eight days then 50 μg. for four days). HCG was then given for 3, 7, or 10 days. After twelve days of estrone priming there was an augmented response to HCG. Payne and Runser (6) found no augmentation to HCG if the estrogen was given concurrently with the HCG (no priming).

Simpson *et al.* found that estrogen priming (eight days) caused augmented responses to pituitary FSH but not to ICSH. Our experiments with estrogen-primed rats (two days) showed good augmentation for Armour's LH but not for Armour's FSH. Studies in progress (Smith and Bradbury) have shown that after estrogen priming there are marked augmentations in ovarian weights when doses of HCG larger than 10 units are administered.

Simpson *et al.* (4) noted that FSH caused the formation of corpora lutea and thecal luteinization of large follicles after eight days of estrogen priming. Paesi (10) found that twelve days of estrogen priming favored luteinization when HCG was given. Payne and Runser (6) found that concomitant administration of estrogen depressed the incidence of corpora lutea in PMS treated rats. In our observations two days of estrogen priming reduced the incidence of corpora lutea. These various observations suggest that the condition of the ovary at the initiation of gonadotrophic stimula-

tion is more decisive than the type of gonadotrophin on subsequent histologic developments. Further study is necessary to determine optimal intervals of estrogen priming to achieve augmentation with each of several gonadotrophins. Intraovarian estrogen pellet implants or unilateral ovarian perfusion with estrogen should also be attempted to demonstrate conclusively whether the effect of estrogen can be localized to the treated ovary.

REFERENCES

1. Mazer, C. S., L. Israel, and B. J. Alpers: *Endocrinology* **20:** 753. 1936.
2. Pencharz, R. I.: *Science* **91:** 554. 1940.
3. Williams, P. C.: *Nature* **145:** 388. 1940.
4. Simpson, M. E., H. M. Evans, H. L. Fraenkel-Conrat, and C. H. Li: *Endocrinology* **28:** 37. 1941.
5. Payne, R. W. and A. A. Hellbaum: *Endocrinology* **57:** 193. 1955.
6. Payne, R. W. and R. H. Runser: *Endocrinology* **62:** 313. 1958.
7. Moore, C. R. and D. Price: *Am. J. Anat.* **50:** 13. 1932.
8. Bradbury, J. T.: *Endocrinology* **41:** 501. 1947.
9. Paesi, F. J. A.: *Acta Endocrinol.* **11:** 251. 1952.
10. Paesi, F. J. A.: *Acta Physiol. Pharmacol. Neerl.* **1:** 304. 1950.

REVERSIBILITY OF THIOURACIL-INDUCED INHIBITION OF INTERPUBIC SEPARATION IN VIRGIN MICE GIVEN THYROXINE OR LARGE DOSES OF RELAXIN[1]

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ABSTRACT

A previous report of markedly reduced pubic symphyseal separation in response to exogenous relaxin in nulliparous mice fed thiouracil is substantiated in this study. The data indicate that the resultant depression of thyroid hormone levels following thiouracil administration does not impart irreparable damage to the mechanism for pelvic relaxation.

Although the withdrawal of dietary thiouracil and the administration of thyroxine are more satisfactory means of diminishing the inhibition of the symphyseal response, a large single dose of a low-potency relaxin preparation nevertheless appears capable of producing symphyseal separation even in the presence of the block.

PARTIAL inhibition of the pubic symphyseal response to exogenous relaxin was noted in nulliparous mice fed thiouracil (0.5%) in a Purina fox chow ration. Similarly, virtually complete inhibition of this response occurred when the drug content of the diet was increased to 1.0% (1). On the other hand it was observed, in the same study, that dietary thyroid powder supplements appeared to enhance the response of the pubic symphysis to relaxin.

Two quite obvious questions arose from these data. The first is with reference to the reversibility of such thiouracil-induced inhibition of pubic separation following drug withdrawal or by employment of larger doses of relaxin. The second concerns a possible stimulatory influence which thyroxine might exert in hypothyroid mice, in addition to restoration of normal thyroid hormone levels, upon the symphyseal response.

The following report deals with the effects of dietary thiouracil withdrawal, of thyroxine therapy, and of large doses of relaxin upon the inhibition of pelvic relaxation in mice previously fed thiouracil.

MATERIALS AND METHODS

Drug Withdrawal and Thyroxine Administration

One hundred and twenty virgin female mice (Albany strain) 100–130 days old

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were fed Purina fox chow and tap water, *ad libitum*, for a period of 30 days, whil another 120 similar mice, identically maintained, ate 1.0% thiouracil[2] added to th basic chow ration. At the end of the 30-day period 30 mice of those fed only fox cho were used to compile Group 1. This group received, subcutaneously, 5 μg. estradiol cyclopentylpropionate[3] immediately (day 30). These animals received 35 μg. relaxin[4] i 0.2 ml. of 1.0% benzopurpurine 4-B[5] on the 38th day and were killed on the 39th day Thirty mice of those fed fox chow with thiouracil were taken to make Group 3 whic was treated identically with Group 1, but remained on the thiouracil diet. These groups are indicated as having 0 days *recovery*.

The remaining mice (90 fed chow only and 90 fed chow and thiouracil) were maintained on their respective diets for 9 additional days (days 30–39) in order to equate the lengths of initial feeding periods with those of Groups 1 and 3. At the end of this period fox chow-fed animals were divided into Groups 5 and 6 to be maintained 10 days, Groups 9 and 10 for 20 days and Groups 13 and 14 for 30 days of *recovery* on the fox chow diet. Groups 6, 10 and 14 received, daily, 10 μg. thyroxine,[6] subcutaneously, throughout their respective *recovery* periods. Animals previously fed thiouracil for 39 days had the drug withdrawn at that time. These mice were divided into Groups 7 and 8 for 10 days, Groups 11 and 12 for 20 days, and Groups 15 and 16 for 30 days of *recovery* on a fox chow diet. Only Groups 8, 12 and 16 received 10 μg. thyroxine, daily, during their respective *recovery* periods. Nine days prior to the end of these various *recovery* periods (days 40, 50 and 60) each animal received, subcutaneously, 5 μg. estradiol-cyclopentylpropionate followed in 8 days by 35 μg. relaxin in benzopurpurine. Autopsies were then performed on the 49th, 59th and 69th days.

High Relaxin Dosage

Virgin mice of the Albany strain (100–130 days old) were divided into 6 major groups of no less than 60 animals. Groups I and V were fed fox chow and tap water, *ad libitum*, Groups II and VI, fox chow plus 1.0% thiouracil, all for a period of 44 days. Group III was fed 0.5% thiouracil and Group IV, 1.0% thiouracil in fox chow, both for an 88 day period. Each animal received, subcutaneously, 5 μg. estradiol-cyclopentylpropionate on day 35 except mice of Groups III and IV which received their estrogen prime on the 79th day.

These 6 major groups were then divided into lesser groups of no less than 15 mice each in order to receive relaxin in dosages of 0.035 mg., 0.1 mg., 1.0 mg. and 5.0 mg. Relaxin was administered in 0.2 ml. benzopurpurine to Groups I–IV and in 0.1 ml. 5% beeswax in peanut oil[7] to Groups V and VI. Injections of relaxin were made on the 43rd day except for mice of Groups III and IV in which case it was day 87. Autopsies were done 24 hours later, thus, on days 44 and 88.

All animals for the entire study were killed with ether and interpubic ligaments were measured directly with vernier calipers following exposure of the symphysis pubis by dissection.

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[2] Nutritional Biochemicals Corp., Cleveland, Ohio.

[3] Depo-estradiol, Upjohn, Kalamazoo, Michigan.

[4] Releasin preparation #80516, Lot M43, each mg. equivalent to 0.12 mg. relaxin Reference Standard W1164-A, lot 8; generously given by Warner-Lambert Research Institute, Morris Plains, New Jersey.

[5] Kindly supplied by Drs. Kroc and Steinetz, Warner-Lambert Research Institute.

[6] Sodium L-Thyroxine, Ref. No. 246-5 generously donated by Dr. Harry Fevold, Travenol Laboratories, Morton Grove, Illinois.

[7] Repository vehicle furnished by Warner-Lambert Research Institute, Morris Plains, New Jersey.

RESULTS

Drug Withdrawal and Thyroxine Administration

Interpubic ligaments of *control* mice (Group 1) measured on the 39th day averaged 1.72 mm. (Table 1). There was no significant deviation from this measurement despite a 10, 20 or 30 day *recovery* period (Groups 5, 9, 13). Thyroxine appeared to be without effect, during the first 20 days of the *recovery* period (Groups 6 and 10), but became effective when given for a period of 20–30 days (Group 13).

The addition of thiouracil (1.0%) to the diet produced the same marked inhibition of response to relaxin previously noted (1). When chow, containing no thiouracil, was substituted on the 39th day for the drug-containing chow, a marked increase in pubic responsiveness to relaxin was noted after 10 days (Group 7), an even greater increase in 20 days (Group 11), and essentially a "normal" response 30 days (Group 15) after drug withdrawal. On the other hand, daily administration of thyroxine during the *recovery* period produced a somewhat greater than "normal" response within a 20-day period (Group 12) and a stimulatory effect, similar to that seen in mice fed thyroid powder (1), was apparent after 30 days (Group 16).

High Relaxin Dosage

Average interpubic ligament length was 1.69 mm. in virgin mice previously fed fox chow and given 0.035 mg. relaxin (Group I) (Table 2). When 0.1 mg. relaxin was administered a significantly greater response occurred. However, increasing the dosage to 1.0 mg. or 5 mg. produced no further significant increase in symphyseal separation than occurred in mice given 0.1 mg. relaxin.

The marked inhibition of the pelvic response to relaxin which occurred in mice fed thiouracil for 44 days (Group II) was reduced by larger doses of relaxin, but even a dose of 5 mg. produced a response only half as great as that seen in *controls* given but 0.035 mg. of relaxin. When the period of drug feeding (at the 1.0% level) was extended to 88 days (Group IV) pubic separation resulting from 5.0 mg. relaxin was only about one-third that of controls given 0.035 mg. Mice fed only 0.5% thiouracil for a similar extended period (Group III) responded considerably better.

Generally, the response of thiouracil-fed mice to relaxin in beeswax and peanut oil (Group VI) was greater than that of similar mice given the hormone in benzopurpurine (Group II). Relative to the appropriate control mice (Groups V and II), however, identical doses of relaxin in the beeswax and peanut oil vehicle produced no greater reduction of the thiouracil-induced inhibition than did relaxin in benzopurpurine.

DISCUSSION

Results of this study further substantiate an earlier report of markedly reduced responsiveness of the pubic symphysis to exogenous relaxin in

TABLE 1. THE INFLUENCE OF DIETARY THIOURACIL WITHDRAWAL AND/OR THYROXINE ADMINISTRATION UPON AVERAGE INTERPUBIC LIGAMENT LENGTHS IN RELAXIN-TREATED FEMALE MICE PREVIOUSLY FED 1% THIOURACIL IN FOX CHOW*

Initial feeding period on fox chow diet		Recovery feeding period on fox chow diet	Average interpubic ligament length in mm.							
Supplement	Days	Daily Injection	Group No.	0 days recovery at 39 days	Group No.	10 days recovery at 49 days	Group No.	20 days recovery at 59 days	Group No.	30 days recovery at 69 days
None	39	None	1	1.72 ± 0.05†	5	1.81 ± 0.16	9	1.80 ± 0.11	13	1.70 ± 0.21
None	39	10 μg. Thyroxine	2	—	6	1.74 ± 0.20	10	1.84 ± 0.09	14	2.56 ± 0.28
1% Thiouracil	39	None	3	0.32 ± 0.08	7	0.82 ± 0.02	11	1.12 ± 0.16	15	1.68 ± 0.17
1% Thiouracil	39	10 μg. Thyroxine	4	—	8	1.16 ± 0.14	12	1.77 ± 0.19	16	2.06 ± 0.14

* All mice received 5 μg. estradiol-cyclopentylpropionate 9 days prior to autopsy (days 30, 40, 50 or 60), 35 μg. relaxin 24 hours before killing (days 38, 48, 58 or 68) and were killed on days 39, 49, 59 or 69.

† Standard error of the mean.

TABLE 2. INFLUENCE OF VARYING DOSAGES OF LOW-POTENCY RELAXIN POWDER AND A COMPARISON OF TWO REPOSITORY VEHICLES UPON AVERAGE INTERPUBIC LIGAMENT LENGTHS IN MICE FED THIOURACIL*

Group	Dietary regimen			Relaxin dosage			
	Diet	Days	Vehicle	.035 mg.	0.10 mg.	1.0 mg.	5.0 mg.
I	Fox Chow	44	Benzopurp.	1.69 ± 0.11† (20)‡	1.91 ± 0.19 (20)	1.96 ± 0.22 (11)	2.03 ± 0.16 (10)
II	Fox Chow + 1.0% Thiouracil	44	Benzopurp.	0.32 ± 0.06 (24)	0.45 ± 0.03 (17)	0.65 ± 0.04 (33)	0.71 ± 0.09 (21)
III	Fox Chow + 0.5% Thiouracil	88	Benzopurp.	—	0.83 ± 0.07 (12)	1.01 ± 0.10 (10)	1.15 ± 0.17 (9)
IV	Fox Chow + 1.0% Thiouracil	88	Benzopurp.	—	0.18 ± 0.02 (5)	0.44 ± 0.03 (5)	0.58 ± 0.06 (7)
V	Fox Chow	44	Beeswax in Oil	1.90 ± 0.16 (7)	2.10 ± 0.22 (8)	2.40 ± 0.20 (15)	2.57 ± 0.23 (13)
VI	Fox Chow + 1.0% Thiouracil	44	Beeswax in Oil	0.30 ± 0.04 (7)	0.59 ± 0.08 (8)	1.08 ± 0.11 (15)	1.20 ± 0.09 (15)

* All mice were primed with 5 μg. estradiol-cyclopentylpropionate on day 35, received one of the various doses of relaxin on day 43 and were killed on day 44 except mice of Groups III and IV which were primed on day 79, received relaxin on day 87 and were killed on the 88th day.

† Standard error of the mean. Average ligament lengths are given in mm.

‡ Numbers within parentheses indicate number of mice.

mice previously fed a diet containing thiouracil (1). In the same report it was indicated that the "influences of thiouracil upon interpubic ligament elongation are not direct drug effects, but rather appear to result from altered thyroid gland activity." Present data indicate that the resultant depression of thyroid hormone release following thiouracil administration does not irreparably impair the mechanism for pelvic relaxation. Indeed, if the drug is withdrawn from the diet and sufficient time permitted for the animal to regain a more "normal" level of thyroid gland activity, the inhibition of pubic separation appears wholly reversible. Such time as is necessary for recovery, after drug withdrawal, of an essentially normal symphyseal response is seemingly reducible by administration of thyroxine, presumably by hastening the return of a euthyroid state. Results further suggest that once the return has been accomplished the animals (Group 14) are similarly capable of demonstrating the increased responsiveness to relaxin previously observed in mildly hyperthyroid nulliparous mice (1). The apparent absence of permanent impairment and the relative ease and rapidity of overcoming thiouracil-induced inhibition of the symphyseal response by drug withdrawal or thyroxine administration tend to deny indirect ovarian mediation and lend some further support to the thesis that the effects of abnormal thyroid activity upon pubic responses to relaxin are direct influences of that gland (1).

Inhibition of interpubic separation following thiouracil feeding appears to be partially ameliorated by high doses of relaxin in benzopurpurine or 5% beeswax in peanut oil as repository vehicles, but the results are somewhat less than satisfactory. Nevertheless, the seemingly excellent responses of the symphysis to relaxin following thiouracil withdrawal and thyroxine administration (present study) were obtained in the absence of the drug block while these latter symphyseal responses to high dosages of relaxin were produced in the presence of such drug inhibition. In view of previous data (1) which indicate that the effects of thiouracil upon pelvic responses to relaxin are not mediated by the ovary it is not surprising that administration of an ovarian hormone (relaxin) is not the most efficacious means of overriding an inhibitory influence seemingly mediated directly by the thyroid gland. However, it is well known that the action of estrogen is impaired in hypothyroid mice. That the defect in response to relaxin of the thiouracil-fed mouse might be a reflection of inadequate estrogen priming is a factor presently under investigation.

It is interesting to note that at all dosage levels of relaxin employed, the degree of thiouracil-induced inhibition (1% thiouracil) was not reduced either by elevation of the hormone dosage or by employment of another repository vehicle such as beeswax in oil. Instead, average interpubic ligament lengths of thiouracil-fed (1%) mice merely paralleled those fed only fox chow (almost constantly a 1.4 mm. difference) (Table 2) at a given relaxin dosage and similar vehicle. However, despite this apparent parallel-

ism it must be conceded that in mice previously fed 1% thiouracil, low-potency relaxin (although large dosages are required) in a repository vehicle (beewax in oil) reportedly half as effective as benzopurpurine (2 did produce a four-fold increase in ligament length (0.32 mm., Group II t 1.20 mm., Group VI). The use of high-potency relaxin preparations or re peated administration of moderate doses of low-potency relaxin powder i combination with highly effective repository vehicles might possibly b even more effective in the amelioration of the inhibitory influences o thiouracil upon pelvic relaxation than the single dose of a low-potenc preparation used in this study.

REFERENCES

1. Horn, E. H.: *Endocrinology* **63**: 481. 1958.
2. Steinetz, B. G., V. L. Beach and R. L. Kroc: Recent Progress in the Endocrinology of Reproduction, pp. 389–427, 1959. Academic Press, New York, N.Y.

NOTES AND COMMENTS

HANGES IN THE THYROID: SERUM RADIOIODIDE CONCENTRATION RATIO DURING THE ESTROUS CYCLE OF THE RAT[1]

ABSTRACT

The thyroid:serum radioiodide concentration (T/S) ratio of cycling female rats is at its lowest level during proestrus and highest during late estrus. T/S values during metestrus, early estrus and diestrus are intermediate and not significantly different from each other.

The results of several investigations have indicated that thyroidal iodine uptake is greater during estrus than any of the other stages of the estrous cycle (1, 2). These authors, however, did studies of thyroid I^{131} uptake during 6 hours after determination of the stage of the estrous cycle. This could possibly mean that the values obtained in some cases would more appropriately reflect conditions during the next stage, since certain stages have a duration measured in hours (3). I^{131} uptake is also a poor measure of thyroid function since it is considerably influenced by extrathyroidal factors.

In this paper thyroid/serum iodide concentration ratios (T/S) were determined simultaneously with the identification of the stage of the cycle. This measurement is much less influenced by factors outside the pituitary-thyroid axis than is percentage uptake of I^{131}.

MATERIAL AND METHODS

Adult Sprague-Dawley, female rats weighing between 225–250 gm. were used. All were fed Purina Laboratory Chow *ad libitum* at least 3 weeks before the measurement of the T/S.

The T/S was determined as follows. Twelve mg. of propylthiouracil (PTU) in 0.6 ml. of alkaline solution were injected subcutaneously forty-five minutes prior to a subcutaneous injection of 5 μc. of carrier-free I^{131} in one ml. of distilled water. One hour later, the animals were anesthetized with ether and blood was obtained from the abdominal aorta. The thyroid was dissected out and weighed on a Roller-Smith torsion balance. One-tenth ml. of serum and the thyroid were prepared for radio-activity determination by the method described by VanderLaan and Greer (4).

Vaginal smears were taken approximately one minute before the rat was sacrified. Only those rats whose vaginal smears could be identified as typical for one of the 5 stages of the estrous cycle (proestrus, early estrus, late estrus, metestrus and diestrus) with absolute certainty were used in the experiment.

RESULTS

The thyroid serum radioiodide concentration ratios during the different stages of the estrous cycle are summarized in Figure 1.

The T/S was found to be lowest during proestrus. With the onset of early estrus the T/S increased, reaching its highest values in late estrus. During metestrus the T/S was not found to be significantly different from that determined during diestrus.

DISCUSSION

The results of this investigation indicate that the ability of the thyroid to concentrate iodide from the serum in the female rat shows cyclic changes which can be correlated with stages of the estrous cycle.

We have found that the T/S of proestrus animals is significantly different from that found in all of the other stages of the estrous cycle. No such change in thyroid function

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during proestrus was observed by Soliman and Reineke (1). The possible reasons for this discrepancy may be that in their rats vaginal smears were not taken simultaneously with thyroid I^{131} uptake measurements, but 6 hours earlier; and that they used thyroidal I^{131} uptake rather than T/S as a measurement of thyroidal activity. Long and Evans (3) have demonstrated that proestrus and early estrus last for approximately 12 hours and metestrus has a duration of only 6 hours. It is conceivable that Soliman and Reineke's (1) animals could have progressed into the next stage of the cycle. An animal in proestrus may have been actually in estrus at the time of thyroidal I^{131} uptake measurements. Thus, false high values could have been recorded for proestrus.

Dividing the stage of estrus into early and late estrus enabled us to demonstrate that thyroidal activity varies significantly even within this period of estrus. Soliman and Reineke's (1) procedure of combining early and late stages of estrus did not permit such a distinction. Also, some of their estrous rats may have been metestrous by the time their I^{131} uptake was determined. This may in part explain why our maximum T/S (during late estrus) was 290% greater than that during proestrus, while Soliman and Reineke found a maximum difference of only 165% between I^{131} uptake in "proestrus" and "estrus."

Feldman (2) reported thyroid I^{131} uptakes for various stages of the cycle, but since

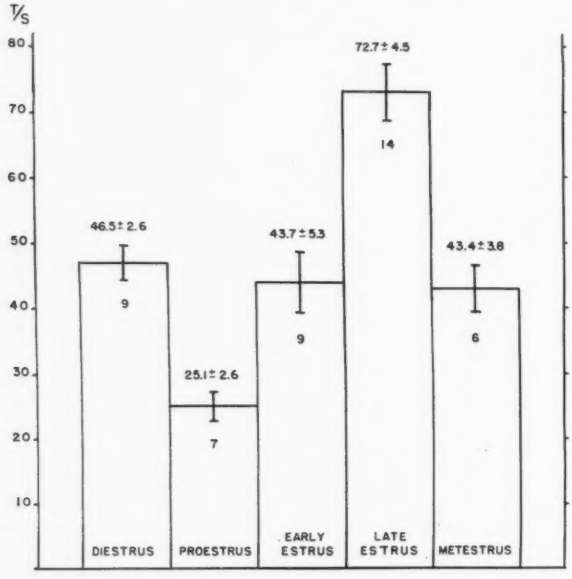

FIG. 1. Thyroid: serum radioiodide concentration ratios (T/S) during the various stages of the estrous cycle. Vertical bars represent mean T/S values, vertical lines standard errors. Figures within bars represent number of rats in the group.

P values

D vs. P <0.01
D vs. eE not significant
D vs. LE <0.01
D vs. M not significant
P vs. eE 0.01–0.02
P vs. lE <0.01
P vs. M <0.01
eE vs. lE <0.01
eE vs. M not significant
lE vs. M <0.01

(D = diestrus, P = proestrus, eE = early estrus, lE = late estrus, M = metestrus.)

he measured the collection of I^{131} for 24, 48 or 72 hours it is hard to understand how he could classify his cycling animals as having been in any particular stage of the cycle while their thyroid took up the I^{131}.

Soliman *et al.* (5) found that the thyrotrophic hormone (TSH) in the blood of rats is t its highest level during estrus. Correlation of this observation with our findings is eset with difficulties. A single subcutaneous injection of TSH does not elevate the T/S f hypophysectomized rats for at least 6 hours, and the maximal effect of a dose of TSH n the iodide pump is not obtained until 1–2 days after the injection (7, 4). Therefore he change in T/S during one stage of the estrous cycle may in fact have been itiated during an earlier stage, since a transitional depression of the T/S preceding s rise has been observed after the injection of TSH into hypophysectomized rats (6). nother possibility is that TSH affects the T/S at least in part through alteration of the ganic iodine concentration in the thyroid (8). However, it is not known whether a bstantial depletion of organic thyroidal iodine occurs during estrus, and if so, whether ch a change affects the T/S promptly.

Preliminary investigations have failed to show any effect of estrogen (5 μg. of stradiol for 3 and 10 days) on the T/S of spayed rats. It is therefore questionable whether a rise of endogenous estrogen production has any role in the elevation of the T/S during late estrus.

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REFERENCES

1. SOLIMAN, F. A. AND E. P. REINEKE: *Am. J. Physiol.* **178:** 89. 1954.
2. FELDMAN, J. D.: *Endocrinology* **58:** 327. 1956.
3. LONG, J. A. AND H. M. EVANS: *Mem. Univ. Calif.* **6:** 1. 1922.
4. VANDERLAAN, W. P. AND M. A. GREER: *Endocrinology* **47:** 36. 1950.
5. SOLIMAN, F. A. AND H. M. BADAWI: *Nature* **177:** 235. 1956.
6. HALMI, N. S.: *Ciba Foundation Colloquia on Endocrinology* **10:** 79. 1956, J. & A. Churchill, Ltd., London.
7. HALMI, N. S., B. N. SPIRTOS, E. M. BOGDANOVE AND H. J. LIPNER: *Endocrinology* **52:** 19. 1953.
8. VANDERLAAN, W. P. AND R. CAPLAN: *Endocrinology* **54:** 437. 1954.

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TRIIODOTHYRONINE UPTAKE BY ERYTHROCYTES

ABSTRACT

Some theoretical considerations and experimental observations concerning the red cell uptake of triiodothyronine-I^{131} are presented.

The uptake by erythrocytes of I^{131}-labeled triiodothyronine (T_3) was studied recently (1, 2, 3) and has been proposed as a diagnostic test of thyroid function. It is still an open question what kind of information is obtained by the red blood cell (RBC) uptake test. Is the uptake a quantitative phenomenon related to the thyroxine-binding capacity of the serum, a metabolic phenomenon, or a combination of both?

Assuming that the red cell uptake of T_3-I^{131} is a physical chemical phenomenon, the following equilibria will be established:

$$P + T_3 \rightleftarrows PT_3 \quad \text{and} \quad E + T_3 \rightleftarrows ET_3$$

where P denotes the T_3 binding sites on the serum proteins and E those in the erythrocytes.

The concentration of free triiodothyronine ($[T_3]_f$) will be the same all over the liquid phase of the system. The law of mass action therefore yields

$$[T_3]_f = k_p \cdot \frac{[PT_3]}{[P_{unsat}]} = k_e \cdot \frac{[ET_3]}{[E_{unsat}]} \qquad (I)$$

The suffix unsat refers to binding sites that are not saturated with T_3 or thyroxine (Tx).

Equation I presupposes that all binding sites on the serum proteins have identical affinities to T_3, and that the same is true of the receptors in the erythrocytes, which is an approximation. The approximation will be better the more constant the relative concentrations of the various thyroid hormone binding proteins are.

When T_3-I^{131} has been added to the system and equilibrium has been established, we determine the ratio (Q) of the amount of T_3 in the erythrocytes to that in the whole system.

Since the concentration of free T_3 is negligible,

$$Q = \frac{[ET_3 - I^{131}]}{[ET_3 - I^{131}] + [PT_3 - I^{131}]} = \frac{[ET_3]}{[ET_3] + [PT_3]} \qquad (II)$$

From this we can calculate

$$\frac{[ET_3]}{[PT_3]} = \frac{Q}{1 - Q}$$

But from (I) we get

$$\frac{[ET_3]}{[PT_3]} = \frac{k_p \cdot [E_{unsat}]}{k_e \cdot [P_{unsat}]} = \frac{k \cdot [E_{unsat}]}{[P_{unsat}]}$$

Hence

$$\frac{Q}{1 - Q} = \frac{k \cdot [E_{unsat}]}{[P_{unsat}]} \qquad (III)$$

Erythrocytes absorb only very small quantities of Tx. This may explain why their content of binding sites unsaturated with Tx and thus accessible to T_3 is high relative to their content of saturated receptors. Consequently, $[E_{unsat}]$ will keep fairly constant as long as the variation in serum PBI does not exceed that observed in a human patient material. Another prerequisite that $[E_{unsat}]$ can be regarded as constant, is that the

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matocrit value is kept within narrow limits. Under those circumstances, the red cell ptake ratio for T_3-I^{131}, may be expressed to a good approximation by

$$\text{RBC}-\text{uptake} = \frac{Q}{1-Q} = \frac{K}{[P_{unsat}]} \qquad \text{(IV)}$$

It should be emphasized that the above expression for the erythrocyte uptake is different from that used by Hamolsky, Stein and Freedberg (2). The magnitude used by these authors for characterization of the erythrocyte uptake is identical with the Q occurring in the above equations.

The correctness of the view that the red cell uptake of T_3-I^{131} is a physical chemical phenomenon depending only on the serum content of free T_3-I^{131}, is suggested by the following experimental facts.

A. The RBC-uptake of T_3-I^{131} was determined in a serum and in the same serum diluted with physiological saline. It was found that as the serum was diluted, the erythrocyte uptake increased as predicted by Equation IV.

B. The pretreatment of the erythrocytes for counting involves a series of washing procedures which might conceivably disturb the equilibrium between the T_3 bound to erythrocytes and that bound to the serum proteins. However, the dilution of the serum by this saline will change P_{unsat} and PT_3 by the same factor. According to Equation I, the concentration of free T_3 will therefore remain unchanged. This explains the reproducibility of the results obtained by the method.

C. The author has made a comparison of the serum content of free non-proteinbound serum thyroxine-I^{131} with the RBC-uptake of T_3-I^{131} from a number of sera. The free Tx-I^{131} and the free T_3-I^{131} in serum will both be inversely proportional to P_{unsat}. The free Tx-I^{131} was determined by a dialysis procedure as described elsewhere (4). Following the addition of I^{131}-labeled thyroxine to a lo ml serum sample, the sample is dialyzed against lo ml of the same serum to which an equal amount of non-radioactive thyroxine has been added. The rate at which the labeled hormone passes through the membrane may be considered proportional to the concentration of free thyroxine.

The free Tx-I^{131} and the erythrocyte uptake were determined simultaneously in a patient and in a normal subject. Some of the results obtained are shown in Table 1. The results show that the ratio of the free Tx-I^{131} for patient to normal

TABLE 1. COMPARISON OF NON-PROTEINBOUND THYROXINE-I^{131} AND RBC UPTAKE OF TRIIODOTHYRONINE-I^{131} FOR DIFFERENT SERA

Serum No.	Diagnosis	Free Tx-I^{131} (per cent of total passing membrane in 24 hours)	Ratio of free Tx-I^{131} for patient to normal	$\frac{Q}{1-Q}$. 100 corrected to 100 per cent hematocrit	Ratio of corrected $\frac{Q}{1-Q}$ for patient to normal
1	Pregnancy	0.31		11.82	
2	Normal	0.48	0.64	18.63	0.63
3	Pregnancy	0.30		10.79	
4	Normal	0.54	0.55	19.69	0.55
5	Myxoedema	0.42		13.80	
6	Normal	0.59	0.71	18.18	0.76
7	Hyperthyroidism	1.38		52.59	
8	Normal	0.52	2.65	20.00	2.63

is in most cases in good agreement with the ratio of RBC-uptake for patient to normal.

This suggests that the erythrocyte uptake of T_3-I^{131} is a quantitative phenomenon that depends only on the serum concentration of unbound T_3-I^{131}.

From paper electrophoretic experiments (5, 6) it is not clear which binding patterns of T_3 and Tx are naturally occurring. Some of the phenomena observed may be artefacts due to the changing in the binding affinity of serum components in the buffer system. The present experiments might indicate that the same binding sites are available to triiodothyronine and thyroxine.

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REFERENCES

1. Crispell, K. R. and J. Coleman: *J. Clin. Invest.* **35:** 1263. 1956.
2. Hamolsky, M. W., M. Stein and A. S. Freedberg: *J. Clin. Endocrinol. and Metab.* **17:** 33. 1957.
3. Hamolsky, M. W., A. Golodetz and A. S. Freedberg: *J. Clin. Endocrinol. and Metab.* **19:** 103. 1959.
4. Christensen, L. K.: *Nature* **183:** 1189. 1959.
5. Ingbar, S. H.: *Endocrinology* **63:** 256. 1958.
6. Tanaka, S. and P. Starr: *Acta Endocrinol.* **31:** 161. 1959.

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MASCULINIZATION OF FEMALE FETUSES IN THE RAT BY PROGESTATIONAL COMPOUNDS

ABSTRACT

Progesterone, 17α-ethynyl-19-nortestosterone, and 6α-methyl-17α-acetoxy progesterone were administered to pregnant female rats and the newborn were examined at birth and at 20 days for evidence of masculinization. Progesterone caused no abnormalities while both synthetic compounds produced pseudohermaphrodism.

Recently Wilkins *et al.* (1) and others (2, 3, 4, 5) have reported numerous cases of human female pseudohermaphrodism following the administration of progestins during gestation. This masculinization may possibly be attributed to the androgenic properties of the steroids since androgens administered during pregnancy are known to cause pseudohermaphrodism in the human (5, 6) and also in laboratory animals. Given at the critical time in the course of pregnancy, these hormones apparently traverse the placental barrier, reach the embryo and modify its sexual development causing masculinization of the female fetuses. Female newborn rabbits with external genitalia resembling the male can be produced by intra-peritoneal implantation of 2 mg. of testosterone propionate on the 19th day of fetal life (7). In rhesus monkeys, female pseudohermaphrodites were born after intramuscular testosterone propionate administration to the pregnant mothers (8). Similar effects of androgenic hormones have been observed in mice (9), hamsters (10), and rats (11).

Our studies were undertaken to determine whether the clinical observations of pseudo-

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hermaphrodism after progestational therapy could be reproduced in experimental animals. The rat seemed to be a suitable animal for this experiment, since although its gestation period is short, embryonic differentiation of the sex glands occurs late in pregnancy. Implantation occurs on the 5th day and the indifferent gonad starts to differentiate into a testis on the 14th day, and into an ovary on the 17th day (12). Hamilton and Wolfe (13) have demonstrated the following changes after the administration of 0.5 mg. of testosterone propionate to rats during the last third of pregnancy; a masculine appearance externally in all young, and in the females enlarged penis-like clitoris, elongated ano-genital distance and a scrotum-like perineum. With higher dosages the perineal orifice of the vagina was missing, the vagina ended blindly, and the uterus was distended with retained secretion. Sometimes a connection was seen between the blind vagina and the urethra.

Similar anomalies have been observed in the experiments described below with the administration of two synthetic progestational compounds.

METHODS

The techniques employed were similar to those described by Hamilton and Wolfe (13). Vaginal smears taken from mated female hooded rats of Long-Evans strain were examined for sperm and animals with positive smears were separated. A further indication of pregnancy was obtained by measuring the gain in body weight on the 15th day. Progesterone (I), 17α-ethynyl-19-nortestosterone (II) and 6α-methyl-17-acetoxy progesterone (III) were administered daily subcutaneously in sesame oil from the 15th to the 20th day of pregnancy. Delivery was delayed after large doses of these compounds, so the pups were removed by Caesarean section on the 22nd day and were placed with foster mothers. On the day of birth the ano-genital distance was measured. Animals which survived to the 20th day were dissected and the reproductive system was carefully examined.

RESULTS

Newborn rats with ano-genital distances between 1 and 2 mm. were considered to be females and those with a distance of 3 to 4 mm. to be males. When the distance was between 2 and 3 mm., the sex could not be determined. The results of progestin treatment during fetal life on the newborn are presented in Table 1. No differences were observed in the ano-genital distances of rats which received progesterone treatment when compared to controls and sex was easily determined. The rats receiving 0.5 mg. of II daily could also easily be sexed by the ano-genital distance. At 1.0 mg. there were 13 intersexuals out of 74 newborn, and at 1.5–2.5 mg. no newborn could be considered female—all were intersexuals or males. At 5.0 and 10.0 mg. all young resembled males. At 0.25 mg. of III, three intersexuals were found out of 38, while at 1.0 and 2.0 mg. only intersexuals and males were seen. At higher doses, 2.5 or 5.0 mg. daily, all newborn were males in their external appearance. Variable enlargement of the clitoris was observed in all the newborn animals treated with II or III and those receiving high doses of I. This effect is related to the androgenicity of the compounds.

The details of the internal sex organs at 20 days of age of the females and female pseudohermaphrodites are presented in Table 2. No abnormalities were found in the sex organs of animals treated with I. At doses of 1 mg. or more of II all females had obvious evidence of masculinization, with either blind vaginas or urethro-vaginal fistulas. At the lowest dose of III, 0.25 mg. daily, it was possible to see masculinization in two females out of 17. At 2.0 and 2.5 mg. all females were pseudohermaphrodites; externally they all appeared like males, and showed either urethro-vaginal fistulas without perineal vaginal orifices, or the caudal part of vagina was missing.

DISCUSSION

Green, Burrill and Ivy (14) found that progesterone in large doses had androgenic properties. According to Lamar, 1 mg. of progesterone has, in castrated male rats, an androgenic potency equivalent to 0.03 mg. of testosterone propionate (15). Since 0.5 mg.

TABLE 1. THE EFFECT OF PROGESTIN TREATMENT DURING FETAL LIFE ON THE SEXUAL CHARACTERISTICS AND BODY WEIGHTS OF 1 DAY OLD RATS

Compound	No. of treated mothers	Daily dose (mg. s.c.)	Litters						Clitoris	Average body weight (gm.)
			Females		Intersexuals		Males			
			No. of young	Anogenital dist. (mm.)	No. of young	Anogenital dist. (mm.)	No. of young	Anogenital dist. (mm.)		
Controls	10	0.2 cc. Sesame oil	46	1.5±0.04			42	3.6±0.06	Normal	6.2±0.13
Progesterone (I)	11	5.0	39	1.4±0.02			69	3.4±0.03	Normal	6.3±0.10
	3	50.0	14	1.8±0.04			19	3.8±0.06	Normal	5.2±0.06
	3	100.0	13	1.8±0.07			20	3.4±0.06	Moderately enlarged	4.7±0.15
	5	200.0	38	1.7±0.03			25	3.2±0.02		5.1±0.07
17α-Ethynyl-19-nortestosterone (II)	5	0.5	20	1.8±0.03			26	3.2±0.02	Enlarged	4.7±0.05
	8	1.0	16	1.6±0.03	13	2.4±0.05	45	3.3±0.03	Enlarged	4.9±0.06
	5	1.5	—		18	2.5±0.04	16	3.1±0.03	Enlarged	5.0±0.07
	8	2.5	—		19	2.5±0.03	55	3.1±0.01	Enlarged	4.1±0.07
	4	5.0	—		—		15	3.1±0.04	Enlarged	3.2±0.09
	3	10.0	—		—		20	3.1±0.03	Enlarged	3.7±0.7
6α-Methyl-17α-acetoxy progesterone (III)	4	0.25	18	1.6±0.05	3	2.6±0.20	17	3.5±0.07	Moderately enlarged	5.9±0.13
	4	1.0	—		14	2.6±0.02	21	3.2±0.06	Moderately enlarged	5.8±0.08
	5	2.0	—		8	2.7±0.04	39	3.0±0.02	Enlarged	5.4±0.06
	4	2.5	—		—		44	3.2±0.03	Enlarged	4.8±0.04
	3	5.0	—		—		30	3.2±0.04	Enlarged	4.4±0.04

TABLE 2. THE EFFECT OF PROGESTIN TREATMENT DURING FETAL LIFE ON 20 DAY OLD RATS WITH FEMALE INTERNAL SEX ORGANS

Compound	Daily dose (mg.)	Number of rats	Normal external and internal sex organs	Blind vagina. No vaginal perineal orifice	Urethro-vaginal fistula. No vaginal perineal orifice
Controls	0.2 cc. Sesame oil	25	25		
Progesterone (I)	5.0	27	27		
	50.0	13	13		
	100.0	7	7		
	200.0	22	22		
17α-Ethynyl-19-nortestosterone (II)	0.5	12	12		
	1.0	15		3	12
	1.5	14		1	13
	2.0	8		8	
	2.5	17		15	2
6α-Methyl-17α-acetoxy progesterone (III)	0.25	17	15		2
	1.0	18	4	8	6
	2.0	12		9	3
	2.5	8			8

of testosterone propionate daily produces pseudohermaphrodism in young female rats, approximately 20 mg. of progesterone would be expected to produce a similar effect. However, in our experiments no pseudohermaphrodism was observed even at daily doses of 200 mg. of progesterone. The masculinization in the 17α-ethynyl-19-nortestosterone treated group was not unexpected, since androgenic activity has been reported for this compound (1). We were rather surprised to find masculinization in the 6α-methyl-17-acetoxy progesterone treated group, since Lyster *et al.* (16) did not find appreciable androgenic properties with this compound in the rat.

The production of pseudohermaphrodism by 6α-methyl-17α-acetoxy progesterone, which according to these workers has little androgenic activity, and the failure of progesterone to cause masculinization, although it has androgenic properties, suggests that these activities are not necessarily parallel in any particular steroid. Tests for masculinizing effects should therefore be included in the studies on new synthetic progestational compounds and it would appear important to develop orally active progestins without these undesirable properties.

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REFERENCES

1 WILKINS, L., H. W. JONES, G. H. HOLMAN AND R. S. STEMPFEL: *J. Clin. Endocrinol. and Metab.* **18:** 559. 1958.

2. Reilly, W. A., D. E. Pickering and J. T. Crane: *Am. J. Dis. Childhood* **95:** 9 1958.
3. Hayles, A. B., R. B. Nolan: *Proc. Staff Meet., Mayo Clin.* **33:** 200. 1958.
4. Hillman, D. A.: *Can. Med. Ass. J.* **80:** 200. 1959.
5. Nellhaus, G.: *New Eng. J. Med.* **258:** 935. 1958.
6. Hayles, A. B., R. B. Nolan: *Proc. Staff Meet., Mayo Clin.* **32:** 41. 1957.
7. Jost, A.: The Comparative Physiology of Reproduction and the Effect of Sex Hormones in Vertebrates, Cambridge University Press, p. 237. 1955.
8. Van Wagenen, G. and I. B. Hamilton: Essays in Biology in Honor of Herbert M. Evans, Berkeley, University of California Press, p. 583, 1943.
9. Turner, C. D., *J. Morphol.* **65:** 353. 1939.
10. Bruner, J. A. and E. Witschi: *Am. J. Anat.* **79:** 293. 1946.
11. Greene, R. R., M. W. Burrill and A. C. Ivy: *Am. J. Anat.* **67:** 305. 1940.
12. Greene, R. R., M. W. Burrill and A. C. Ivy: *Am. J. Obst. and Gyn.* **36:** 1038. 1938.
13. Hamilton, J. B. and J. M. Wolfe: *Anat. Rec.* **70:** 433. 1938.
14. Greene, R. R., M. W. Burrill and A. C. Ivy: *Endocrinology* **24:** 351. 1939.
15. Lamar, J. K.: *Anat. Rec.* **70:** Suppl. 45. 1938.
16. Lyster, Stanley, C., G. H. Lund, W. E. Dulin and R. O. Stafford: *Proc. Soc. Exp. Biol. and Med.* **100:** 540. 1959.

DYNAMIC CHANGES IN ASCORBIC ACID AND CORTICOSTEROIDS IN ADRENAL VEIN BLOOD AFTER ACTH[1]

ABSTRACT

Measurement of Ascorbic Acid (AA) and Corticosterone (Compound B) in serial fractions of adrenal vein blood from hypophysectomized rats has demonstrated, immediately after small doses of ACTH, an initial rise in AA, followed by a steady rise in B. As Compound B is rising, AA rapidly falls below pre-ACTH levels. AA release has not occurred in the absence of Compound B release.

The physiologic significance of the high concentration of ascorbic acid (AA) in the adrenal cortex of vertebrates has not been satisfactorily explained. The ability of pituitary adrenocorticotropin (ACTH) to deplete the adrenal of AA (1) and stimulate the synthesis of adrenal cortical steroids in several animal species has given rise to a number of assay procedures for ACTH, and prompted considerable speculation concerning the relationship of AA to steroid biosynthesis. While ascorbic acid depletion and steroidogenesis represent effects of ACTH on the adrenal cortex not shared by other compounds at physiologic concentrations, there as yet appears to be no proof of a direct association between adrenal ascorbic acid and the synthesis or release of steroids.

Recently, two new assays for ACTH have been proposed, based upon the release of ascorbic acid (2) and corticosterone (Compound B) (3) into the adrenal vein of the hypophysectomized rat, and it has been reported that ascorbic acid release into the adrenal vein may precede corticosteroid release after ACTH (4). The minute to minute change in secretion of these substances has not been studied, however, during the critical few minutes immediately after ACTH administration. In the work reported here, AA and Compound B have been measured simultaneously in consecutive minute samples from the adrenal vein of the hypophysectomized rat.

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Male albino rats of approximately 400 gm. were hypophysectomized under ether anesthesia and at varying time intervals thereafter renaesthetized, the jugular vein exposed, the animal heparinized, and the left adrenal vein cannulated. Exactly-timed 1-second serial collections of adrenal vein blood were obtained in small vials containing 0.3 ml. saline, and the saline-diluted blood was analyzed for ascorbic acid by a micro-modification of the method of Roe and Kuether (5, 6), and for corticosterone by the fluorescence technique of Silber *et al.* (7). AA was determined in 0.05 ml. of the blood-saline mixture and Compound B in 0.2 ml. of this mixture. Values for both compounds are expressed in μg. per ml. of blood-saline mixture in the one minute collection. USP Reference Standard Corticotropin was administered rapidly by the jugular vein in doses not exceeding 1.0 mU. Several control periods were obtained before ACTH was administered.

Figure 1 demonstrates the findings in a representative animal hypophysectomized 24 hours previously. Timed adrenal vein collections were made before, during, and for 11 minutes after 0.2 mU. ACTH. Ascorbic acid (solid line) and Corticosterone (dotted line) are shown for each minute of the experiment. After the systemic administration of ACTH, AA had increased in adrenal vein blood by the second minute, while steroid increase did not begin until the fourth minute. It can be seen, therefore, that ascorbic acid release preceded corticosteroid release. Of additional interest, however, as corticosteroid levels rose, ascorbic acid began to fall, and fell below the pre-ACTH level. This pattern of response has been observed in six animals, values for an additional two animals being shown in Table 1 (II, III).

Under the conditions of this experiment, steroid release has never preceded ascorbic acid release, nor has ascorbic acid release occurred in the absence of steroid release.

The appearance of ascorbic acid in the adrenal venous effluent would appear to be a more sensitive measure of ACTH effect on the adrenal gland than ascorbic acid depletion (2). Similarly, the appearance of corticosteroids in the adrenal venous effluent is a more sensitive measure of ACTH effect on the adrenal gland than the measurement of peripheral levels of these steroids. Simultaneous measurement of the two in a rigidly controlled timed sequence of adrenal venous bleeding has demonstrated that, during the

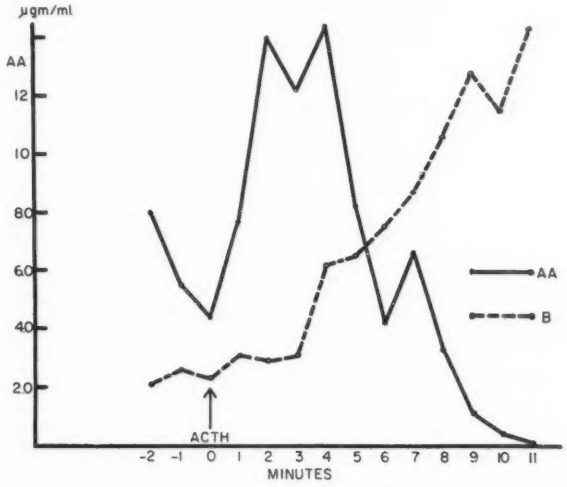

FIG. 1. Secretion of ascorbic acid (AA) and corticosterone (B) in the adrenal vein of a hypophysectomized rat following ACTH administration.

TABLE 1. ASCORBIC ACID AND CORTICOSTERONE IN CONSECUTIVE 60-SECOND COLLECTIONS OF ADRENAL VEIN BLOOD FROM RATS 4 TO 24 HOURS AFTER HYPOPHYSECTOJMY

ACTH in varying doses (I: 0.2 MU.), (II: 0.5 MU.), and (III: 1.0 MU.) given at time 0. Each pair of columns represent absolute values in one large (approximately 400 gm.) albino rat, expressed as μg. (of AA or Compound B) per ml. of blood-saline mixture.

Minutes	I		II		III	
	AA	B	AA	B	AA	B
	μg./ml.		μg./ml.		μg./ml.	
−3			7.14	0.22	8.97	0.31
−2	8.01	0.21	6.89	0.26	0.00	0.28
−1	5.50	0.26	6.89	0.25	6.12	0.25
0 (ACTH)	4.41	0.23	8.31	0.22	1.42	0.28
1	7.71	0.31	2.62	0.19	10.8	0.34
2	14.0	0.29	10.7	0.22	12.6	1.06
3	12.2	0.31	5.91	0.26	14.7	1.87
4	14.4	0.62	12.1	1.09	2.04	2.00
5	8.22	0.65	9.55	1.40	0.00	1.87
6	4.22	0.75	10.7	1.46	0.00	2.68
7	6.67	0.87	12.4	1.75	0.00	3.12
8	3.33	1.06	7.12	2.03	0.00	2.62
9	1.11	1.28	5.95	1.90	0.00	2.93
10	0.44	1.15	3.83	2.03	0.00	2.50
11	0.00	1.43	5.01	2.18	0.00	2.87
12			1.62	—		
13			0.00	1.78		
14			2.42	1.90		
15			2.83	1.62		

first fifteen minutes following the systemic administration of ACTH to the hypophysectomized rat:

a. Ascorbic acid release precedes corticosteroid release.

b. Ascorbic acid levels fall, as steroid levels are rising.

c. No significant AA release has been observed in the absence of steroid release.

The temporal relationship demonstrated here, and the apparent consistency of this sequence of events suggest that it may be worth while to re-investigate the possible role which adrenal ascorbic acid might play in the synthesis and/or release of adrenal cortical steroids in response to ACTH.

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REFERENCES

1. SAYERS, G., M. A. SAYERS, T. LIANG AND C. N. H. LONG: *Endocrinology* **38:** 1. 1946.
2. MUNSON, P. L. AND W. TOEPEL: *Endocrinology* **63:** 785. 1958.
3. LIPSCOMB, H. S. AND D. H. NELSON: *Fed. Proc.* **18:** 95. 1959.
4. SLUSHER, M. AND S. ROBERTS: *Endocrinology* **61:** 98. 1957.
5. ROE, J. H. AND C. A. KUETHER: *J. Biol. Chem.* **147:** 399. 1943
6. RAIHA, N.: *Acta Physiol. Scandinav.* **45,** Supplementum 155, p. 12. 1958.
7. SILBER, R. H., R. D. BUSH AND R. OSLAPAS. *Clin. Chem.* **4:** 278. 1958.

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Endocrinology Study Section
Division of Research Grants
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1.	Hydrocortisone 4-C^{14}	ES-F-10-1	8.7 μc./mg.
	Hydrocortisone 4-C^{14}	ES-F-25-1	21.7 μc./mg.
	Hydrocortisone 4-C^{14}	ES-F-50-1	42.4 μc./mg.
2.	Corticosterone 4-C^{14}	ES-B-10-1	9.1 μc./mg.
	Corticosterone 4-C^{14}	ES-B-50-1	44.4 μc./mg.

These materials are stored at Tracerlab, Inc., Waltham, Massachusetts. They are dispensed in sealed glass ampules containing 5 μc., 10 μc., or 25 μc. each, and in a solution of 95% benzene and 5% methanol.

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